Original article

DOI: http://doi.org/10.20914/2310-1202-2018-3-153-163

A combination of rapid and easy assays of biosurfactant producing bacterial strain isolated from automobiles repairing workshop in Aligarh

Touseef Hussain [1] Hussaintouseef@yahoo.co.in
Abrar Ahmad Khan [1] Khanab2009@gmail.com

[1] Aligarh Muslim University, Aligarh 202002, U.P, India

Summary.Routine washing, cleaning, repairing, maintenance of cars, bikes, scooters and disposal of waste of all kinds are carried out in automobile workshops are common observations in Aligarh. Considering the likelihood of existence of biosurfactant producing organisms at hydrocarbon contaminated site, a large number of soil samples were collected and isolation was carried out. A total of ten bacterial strains ALIG (01–10) were isolated out of which only isolate ALIG01 grown on GSP agar, Maconkey agar as well as on Pseudomonas agar plates which indicated suspected *Pseudomonas spp*. and exhibits positive biosurfactant activity through penetration assay, oil spreading technique, beta hemolytic activity and EI24 (96%), positive blue plate agar plate (> 2cm), qualatitative analysis, tolerance against hydrocarbon *m*-xylene, and microplate assay. This isolate ALIG01 is a valuable source to investigate further for future agriculture plant pathology and industrial applications.

Keywords:automobile, biosurfactant, hydrocarbon, oil contamination

Introduction

Now a-days automobile reparing workshops are creating many different types of hazardous wastes generated during regular services (motarbikes/scooters/cars/lorry/truck) which predominantly include used waste oil and fluids [2]. Such wastes are routinely disposed within the vicinity of workshops. Biodegradation is an effective way to overcome this problem in which microbes utilize the contaminants as a carbon source, leading to the breakdown of the pollution components into low molecular weight compounds [43, 60, 68]. Oil contaminated regions are reported to be a suitable place for the isolation of biosurfactant producing bacterial strains [19]. Microorganisms like bacteria synthesize broad range of surface-active compounds, known as biosurfactants [23]. These are amphilic molecules having both hydrophobic and hydrophilic moieties making them to interfere at different interfaces between the fluids having unlike polarities like hydrocarbons and water [25, 26, 65]. Biosurfactants are a leading group of valuable microbial natural products with unique biochemical properties. From a biotechnology prospective, the production of biosurfactants is important owing to their vast applications in food, cosmetics, pharmaceuticals, and the agricultural and petrochemical industries [6, 12, 27, 38, 50].

However, chemically synthesized surfactant compounds are usually of toxic in nature and are non-biodegradable [61] due to such problems the microbially synthesized surfactants gain more attention as they are eco-friendly and biodegradable [61], biosurfactants, natural products of a variety of microorganisms [44, 46], appear to be preferable for environmental applications. Environmental concerns and stringent regulations have pushed forward the development of natural bio-surfactants as an alternative to chemically synthesized products. During the last few years, a much wider attention has been paid to the isolation of new biosurfactant producing microorganisms from different oil contaminated sites. They have affinity for both polar and non-polar media. Their several advantages over their chemical counterparts include mild production conditions from inexpensive substances [34] lower toxicity [45], better environmental compatibility and biodegradability [21,69]. They possess the property of retaining their activity at extremes of temperature, pH and salt concentration [9]. They exhibit antibacterial, antifungal, and antiviral properties as well as anti-adhesive action against several pathogenic microorganisms [10, 13, 50, 54, 59] They also find application in enhanced oil recovery [20] and bioremediation [54,14] For efficient isolation screening and detection of potential biosurfactant producers isolates, a combination of different (thirteen) easy to screening methods were used, which were successfully evaluated in the present study.

Materials and Methods

Survey and Sample Collection

Soil samples contaminated with waste oil products were collected (beneath 5cm of surface) from near an old Automobile repairing workshop (more than 30 years old shop) near Aligarh Muslim

Для цитирования

Touseef Hussain, Abrar Ahmad Khan A combination of rapid and easy assays of biosurfactant producing bacterial strain isolated from automobiles repairing workshop in Aligarh//Вестник ВГУИТ.2018. Т 80. № 3. С. 153–163. doi:10.20914/2310-1202-2018-3-153-163

For citation

Touseef Hussain, Abrar Ahmad Khan A combination of rapid and easy assays of biosurfactant producing bacterial strain isolated from automobiles repairing workshop in Aligarh. *Vestnik VGUIT* [Proceedings of VSUET]. 2018. vol. 80no. 3. pp. 153–163.. doi:10.20914/2310-1202-2018-3-153-163

University, Aligarh (27.88°№ 78.08°E) during 2017. All the soil samples were collected in clean plastic bags. These samples were used immediately to prevent any deterioration. The sampling site can be seen in Fig. 1.

Figure 1. Sampling site from an automobile workshop

Isolation and Screening of biosurfactants bacterial isolates

5 gm of oil contaminated soil sample from automobile repair workshop was inoculated in 100 ml minimal medium [30] and incubated at 37 ºC, at 200rpm for 48 hours. From that 100ml stock serial dilution technique was used for isolation of biosurfactant bacteria, 1 ml from the soil suspension at (10^2 to $10^{7)}$ dilution was spread on each petri plates of RA2 agar medium (Himedia, Mumbai) and these plates were incubated at 37 °C for 48 hr dark bacterial incubator. Morphologically different colonies were picked/selected and purified on Nutrient agar medium plates (with 0.1% petrol mixed) [55]. The selected bacterial isolates were stored in nutrient agar (Himedia, Mumbai) petriplates as well as on nutrient agar slants (with 0.1% petrol mixed) slants and kept under 4ºC.

Identification and Characterization of isolated bacteria's

Study of Colonial Morphology

Isolated colonies of purified bacterial isolates were grown on solidified Nutrient agar plates (with 0.1% petrol mixed) were observed and data were recorded regarding the form (circular, filamentous, or irregular), elevation (flat, convex, or umbonate), margin (entire, undulate, erose, or filamentous), and optical features (opaque, translucent, or transparent) of the colonies [39].

Cellular Morphology

Cells were observed with Gram staining kit (Himedia, Mumbai) [Duguid, 15] under a microscope (oil immersion, 100×). Shape of the cells (cocci, bacilli, and coccobacilli) and arrangement of cells (scattered, bunches, and chain) along with the Gram reaction were observed.

Lactose Fermentation

Lactose fermenting character in all the isolates was determined by streaking fresh broth culture on MacConkey agar (Oxoid) plates [36]. Results were noted after incubation of 24 h at 37 °C dark incubator.

Pseudomonas Agar Test

The cultures were streaked on Pseudomonas agar plates and incubated at 37ºC for 24–48h in dark incubator and observed under UV transilluminater.

Identification through GSP Agar

The cultures were streaked on Glutamate Starch Phenol Red (GSP) agar plate and incubated at 37 ºC for 24–48 h in dark incubator [28].

Screening Methods for Biosurfactant Production

The isolated bacteria were tested further for biosurfactant production through following methods:

Oil Spreading Method (OSM)

For this test, oil (Petrol) was layered over water in a petriplate (9cm). 10µl supernatant (from culture broth) was added to the surface of oil (petrol) as described by (Morikawa et al. [33]. Occurrence of clear zone was an indication of biosurfactant production. Water drop was used as a negative control [32].

Hemolytic Activity (HA)

The pure culture of bacterial isolates (24h) were streaked on the freshly Sheep blood agar plates (Potous Medium, New Delhi) and incubated at 37 °C for 24–48 h to assay haemolytic activity. Hemolytic activity was detected with the presence of a clear zone (Hemolysis) around bacterial colonies. The plates were visually inspected for clear zones of clearing around colonies. Complete and incomplete hemolysis was designated as (alpha) and (beta) hemolytic activity [52].

Emulsification Index (EI24%)

The emulsifying capacity was evaluated by an emulsification index (EI24%). The EI24% of samples was determined by adding 2 ml of petrol and 2 ml of the cell-free broth in test tube, vortexed at high speed for 2 min and allowed to stand for 24h. The percentage of emulsification index calculated by using the following equation [71].

$$EI24 = \frac{\text{Height of emulsion formed}}{\text{Total height of solution}} \times 100$$

Penetration Assay (PA)

This method relies on the phenomenon of silica gel entering the hydrophilic phase from hydrophobic paste much more quickly in the presence of biosurfactants which leads to a colour change [64]. In this assay, the cavities of a 96 well microtiter plate (Corning, USA) were filled with 150µl of a hydrophobic paste made up of oil (petrol) and silica gel. The paste was covered with 20µl of oil. The 10µl of 1% safranin was added to 90µl of the culture supernatant. In the control, uninoculated medium was added instead of culture supernatant. This coloured supernatant was then placed on the surface of the paste. The upper phase changes from clear red to cloudy white within 15minutes if biosurfactant is present. Biosurfactant free supernatant was turned cloudy but stayed red. Based on the results of qualitative screening tests, positive isolates showing good surfactant activity were selected for further studies.

Blue Agar Plate (BAP) method

Mineral salt agar medium supplemented with glycerol (2%), Cetyltrimethyl ammonium bromide (0.05%) and methylene blue (0.2mg/ml)) was used for the detection of anionic glycolipid [56]. Wells of 5mm diameter were made using cork borer on methylene blue agar plates and loaded with 100µl of fresh culture of individual isolate. The plates were incubated at 37 °C for 48–72h. A dark blue halo zone around the culture was considered positive for anionic biosurfactant production.

Agar Well Diffusion (AWD) method

The ionic property of cell bound biosurfactant producing strain was determined by using agar well diffusion method [29]. Briefly, 3 uniformly spaced wells were made on a soft agar (1%) plate (Himedia, Mumbai), central well was filled with 10µl of suspected ALIG01 isolate biosurfactant producing cell free culture (24hr grown) in Nutrient broth. Either sides of wells were filled with anionic compound (Sodium dodecyl sulfate, 20 mM) and cationic compound (Cetyl trimethyl ammonium bromide (CTAB), 20 mM). Plates were incubated at 25 °C for 24 h and observed for the precipitation lines.

Tolerance Against Hydrocarbon (TAH)

In order to analyze the ability of ALIG01 isolate to grow in presence of hydrocarbon [52], On solidified Nutrient agar plates were coated with 2ml hexane, fresh overnight cultures were spreaded followed by incubation at 37ºC for 24–48 h. Plates were sealed using parafilm-M to avoid evaporation of hexane from petri dishes. A colony surrounded by emulsified halos was considered positive for biosurfactant production.

BATH assay

Bacterial adhesion to hydrocarbons (BATH) Cell hydrophobicity was measured by bacterial adherence to hydrocarbons (BATH) according to a method similar to that described by Rosenberg [49]. The cells were washed twice and suspended in a buffer salt solution (g/l 16.9 K_2HPO_4, 7.3 KH_2PO_4) to give an OD at 600 nm of ~0.5. The cell suspension (2 ml) with 100µl crude oil added was vortex-shaken for 3 min in test tubes (25 x 150 mm). After shaking, crude oil and aqueous phase were allowed to separate for 1 h. The OD of the aqueous phase was then measured at 600 nm in a spectrophotometer. Hydrophobicity is expressed as the percentage of cell adherence to crude oil calculated as follows:

100 x (1 – OD of the aqueous phase/OD of the initial cell suspension).

Modified Drop Collapse (MDC) test

Suspected bacterial isolates was cultured in Nutrient broth medium (Himedia, Mumbai) with 0.1% oil (Petrol) for 48 h. Screening of biosurfactant production was performed using the qualitative drop-collapse test described by Jain [24] as modified by Bodour and Maier [8]. 10µl of gulf oil (engine oil lubricant) was applied to the well regions delimited on the covers of 96-well microplates (Corning, USA) and these were left to equilibrate for 24 h. 5µl of the 48h culture of ALIG01 isolate, after centrifugation at 12,000 rpm for 5 min to remove cells, was transferred to the oil-coated well regions and drop size was observed 1 min later with the aid of a magnifying glass. A result was considered positive for biosurfactant production when the drop diameter was at least 1 mm larger than that produced by deionized water (negative control).

Tilted Glass Slide (TGS) test

Using a sterile toothpick, a fragment of a colony was mixed with a droplet of 0.9% water solution of NaCI on a glass surface (a 26 x 76 mm microscope-slide). If the surface tension decreased enough to allow the water to flow over the surface, the glass-slide test was regarded as positive and the isolates was selected for further testing. Positive strains were maintained on nutrient agar (Himedia, Mumbai) supplemented with 0.1% oil (petrol) per liter; stored at 4 °C and sub cultured every month [47].

Emulsification assay (EU/ml)

Cell-free culture broth was used as the biosurfactant source to check the emulsification of oil (petrol). First, 1 ml of cell-free culture broth was added to 5ml of 50 mM Tris buffer (pH 8.0) in a 30-ml screw-capped test tube. Five milliliters of hydrocarbon was added to the above solution and

vortex-shaken for 1 min, and the emulsion mixture was allowed to remain upright for 20 min. The absorbance of the aqueous phase was measured by spectrophotometer at wavelength of 400 nm. Emulsification activity per milliliter (EU/ml) was calculated by using the following formula:

1 emulsification unit = 0.01 OD400 • dilution factor [48]. A negative control was maintained with only buffer solution.

Quantitative Microplate (QM) Analysis

The surfactant activity of selected ALIG01 isolate was determined using a multiwell plate assay. A 100 µl sample was taken from the supernatant of ALIG01 isolate and was added to a microwell of a 96-microwell plate (Corning, USA). The plate was then viewed using a backing sheet of paper with a black and white grid. The optical distortion of the grid provided a qualitative assay for the presence of surfactant.

Parafilm M Test

A 25 µl aliquot of culture supernatant was dropped on a strip of parafilm M (4 inH125 ft) as a hydrophobic surface and the shape of the drop on the surface was inspected after 1 min. then the diameter of the droplet was evaluated [53]. The phosphate buffer (pH 7.0) were used as negative control, respectively [22,31].

Results

Isolation of Bacterial Population

Total, ten unique different aerobic bacterial population were isolated from oil contaminated soil samples collected from automobile repairing workshop near AMU campus, Aligarh. Repeated streaking and sub-culturing of these bacterial isolates were purified and screened for the initial biosurfactant production bacteria test on specific growth media and after that for the biosurfactant production conformation tests. Those isolates which exhibits best biosurfactant activity were carried for further screening and were maintained at 4ºC.

Identification and Characterization of Isolates Colonial Morphology

The colonial morphology of all these isolates was observed: ALIG01 and 04 were pinpointed, while the rest of the isolates had equal proportions of regular and irregular margins with variable sizes. Furthermore, ALIG02, 06, 10 isolates had convex, ALIG03, 04, 05, 07 isolates concave, and ALIG01, 08, 09 flat elevation of the colony. Most of the colonies had a smooth and shiny texture while only ALIG08, 09, 10 had rough texture. Finally, ALIG01–07 was opaque.

Cellular Morphology

Cellular morphology, such as arrangement, shape, and Gram reaction, was observed: ALIG02–05, 09, 10 were cocci and the rest of the isolates were rods. Furthermore, ALIG07, 09, 10 had scattered cellular arrangement, ALIG05–10 were in bunches, and ALIG 01, 02, 04 were arranged in chains. Most interesting were the Gram reactions, which revealed that only ALIG 01, 04 isolates were gram negative and ALIG02, 03–10 were gram-positive.

Lactose Fermentation

Growth on MacConkey agar plates indicated that only ALIG01 isolates had lactose-fermenting capability while other isolates ALIG(02–10) were non lactose-fermenting colonies that appeared transparent and colourless.

Pseudomonas Agar Test

Only ALIG01 isolate was showing fluorescence under UV transillunimater, which was initially identified as Pseudomonas spp. while rest other ALIG (02–10) isolates did not show any fluorescence as seen in Fig.2a.

Identification Through GSP Agar

ALIG01 isolate was found to be surrounded by red-violet zone on GSP medium, accordingly, revealing confirmation of Pseudomonas spp. while rest other ALIG(02–10) isolates showed partial or not able to grow on GSP agar plates Fig.2b.

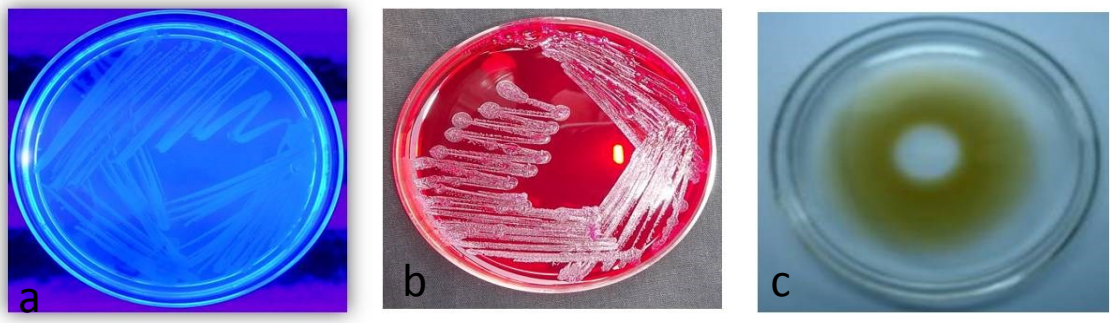


Figure 2. Results of (a) Pesudomonas agar test, (b) GSP agar plate and (c) Oil displacemnt with ALIG01 isolate

Screening for Biosurfactant Production

Oil Spreading Technique

Overnight culture of ALIG01 isolated bacteria were centrifuged and added to oil (petrol) containing periplate. ALIG01 isolate showed the clear zone (2.3cm) by being able to displace the oil (petrol) around the colony indicating biosurfactant production Fig.2c. Rest other ALIG(02–10) isolates showed partial activity (between 0.3–0.5cm). No clear zone was observed with control (water).

Hemolytic Activity

The hemolytic activity was observed in only ALIG01 isolate, results showed positive hemolytic activity (complete clear zones of more than 2cm) while other rest of isolates ALIG(02–10) showed partial activity which were not considered for further study Fig. 2d.

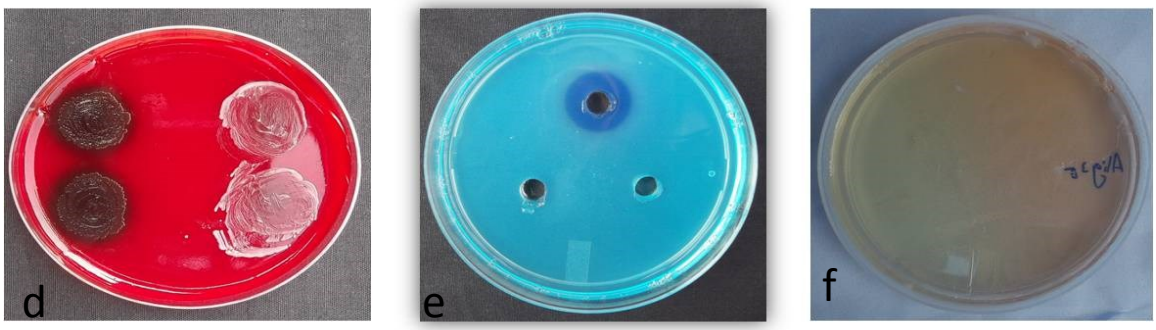


Figure 2. Continued. (d) hemolytic activity, (e) blue agar plate showing blue halos and (f) tolerance against x-ylene with ALIG01 isolate

Emulsification Index (EI24%)

Emulsification index of 50% or more was considered as significantly positive emulsification activity. Our study revealed that among ten bacterial isolates only ALIG01 isolate showed 79% positive emulsification activity with hexane, showed 57% positive emulsification activity with xylene, and 95% were miscible with oil (petrol). Meanwhile, ALIG (02–10) of the isolates showed from partial emulsification with 3 hydrocarbons tested.

Penetration Assay

Only ALIG01 isolate among rest other isolates showed positive penetration test, while rest others isolates did showed partial penetration activity as seen in Fig.3.

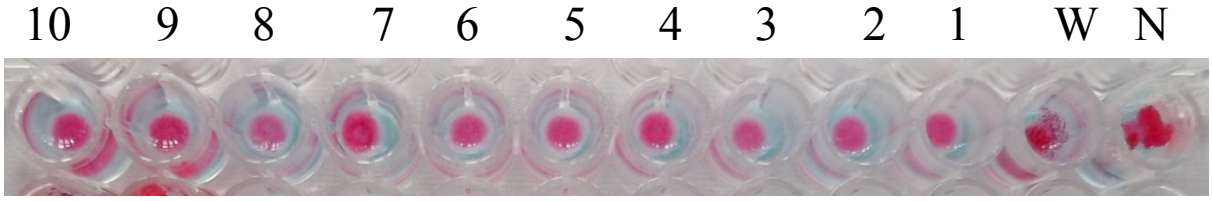


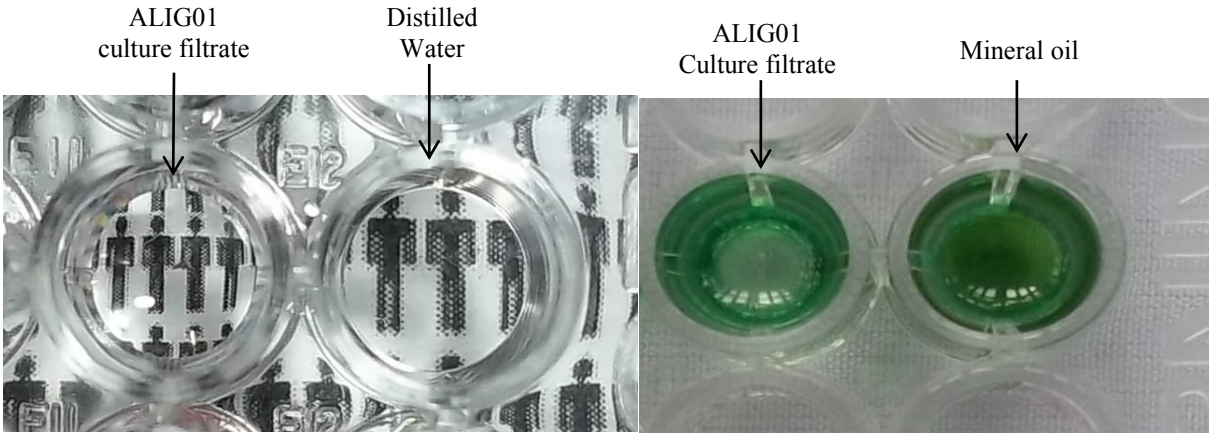
Figure 3. Penetration assay for the sceening of biosurfactants prodcuing isolates. Lane 1 – 10 isolates from oil contaminated site coded as ALIG, W = control, N = negative control

Blue Agar Plates

Only ALIG01 isolate produced dark blue halo around colony (2.2cm) which was considered positive activity for biosurfactant production on blue agar plates as qualitative mark for biosurfactant as shown in (Fig.3e) rest other isolates ALIG(02–10) did not form any blue zones were considered negative.

Agar Double Diffusion Test

Agar double diffusion test which is based on the passive diffusion of two compounds bearing charges of the same or opposite types in a weakly-concentrated gel, revealed precipitation lines between the biosurfactant produced by ALIG01 (suspected Pseudomonas sp.) and the cationic compounds selected (CTAB), while no lines formed between the biosurfactant and the anionic compounds (SDS) as seen in Fig.4.



(a) (b)

Figure 4. **(a)**Qualitative microplate assay with double distilled water and ALIG01 isolate **(b)**Qualitative drop collapse method in Gulf oil engine oil and mineral Oil)

Tolerance Against Hydrocarbon

Heavy growth was observed after 24 h of incubation in only ALIG01 strains which showed tolerance against hydrocarbon (Hexane) rest other did show any tolerance Fig.2f.

BATH Assay

Hydrophobicity of the cell surface as estimated through BATH assay of ALIG01 isolate revealed a maximum mean value of 22.5 ± 2.2% for hexane, followed by 19.1 ± 2.0% for xylene and 17.9 ± 3.2% for oil (Petrol).

Drop Collapse Assay

Only three bacterial isolates ALIG01, 03, 06 were giving positive results in drop collapse test (Fig.4b) while rest isolates ALIG 02, 04, 05–10 showed negative activity.

Tilted Glass Test

Flow of water over the surface of the tilted slide was recorded as positive. Retention of water over the surface, even if the slide was tilted, was considered as negative. ALIG (01, 03, 04, 06) isolates showed a positive test. The other isolates ALIG (02, 05, 07–10) exhibited a negative test as the drop remained stationary on the tilted slide.

Emulsification assay (EU/ml)

Emulsification assay of ALIG01 isolate was performed with three different hydrocarbons: oil (petrol), hexane and xylene. Average emulsification units (EU/ml) results for hexane of 110 ± 5 EU/ml, for xylene of 240 ± 6 EU/ml, and for oil of 186 ± 9 EU/ml were recorded rest others ALIG02–10 isolates showed partial to no activity.

Qualitative Microplate Analysis

We have used a simple microplate assay based on the proposal of Vaux and Cottingham [62], the principle of which is as follows. Pure water in a well has a flat surface which meets the sides of the well at 90°. The presence of surfactant in the water causes some wetting at the edge of the well, and the fluid surface becomes concave as observed in case of ALIG01 isolate. The fluid takes the shape of a single diverging lens, which distorts the image of the grid below the well, when viewed from above (Fig. 4a).

Parafilm M Test

Results showed that the droplet diameter of cell-free supernantant of ALIG01 isolate tested was larger (> 1.8cm) than among other isolates which were considered negative. This indicated that ALIG01 isolate tested produce assayable biosurfactant activity.

Discussion

Globally now a days, various types of activities, including agriculture, industry and transportation produce a large amount of wastes and new types of pollutants. Since antiquity, soil has been the repository of society's wastes [18]. Waste motor lubricant oil is waste oil drained from geared motor vehicles after a long run, which contains weathered hydrocarbon fractions and may be useful for biosurfactant production [1]. The present investigation was conducted with the following objectives: screening of Pseudomonas spp. for biosurfactant production, optimization of biosurfactant production, waste motor lubricant oil and characterization of biosurfactant and estimation of emulsification activity.

At initial screening, isolation and purification process ten different bacteria were isolated, selection was done randomly revealing multiple types of colonies and cellular morphologically. Only those isolates which show biosurfactant activity from initial test screen were screened with other parameters test. Pre-dominantly most of the isolates were gram negative only one isolate ALIG01 was lactose fermenting (identified as Pseudomonas spp.)

because lactose provides a source of fermentable carbohydrate, allowing for differentiation. Similarly another test was followed by through GPS agar plates and Pseudomonas agar plates confirming isolate ALIG01 as Pseudomonas spp. in Fig.2a and 2b. Our study is in agreement with previous findings by Shoeb et al. [77,81]. Further the selected isolate were screened for biosurfactant activity for conformation.

Ability of some strains to show positive biosurfactant production following one method and negative following other methods makes it very difficult to confirm biosurfactant production using two or three methods. In view of this, several screening methods were considered in order to identify the potential organism that can produce biosurfactant. Compared to other isolates, ALIG01 isolate (suspected Pseudomonasspp.)showed better potential in the entire screening test for biosurfactant production. Hence, it's chosen as a promising organism for biosurfactant production based on this study (Table 1).

Table 1.

Comparative analysis of different bio surfactant screening methods

Isolates / Methods	ALIG01	ALIG02	ALIG03	ALIG04	ALIG05	ALIG06	ALIG07	ALIG08	ALIG09	ALIG10
OSM	+++	–	–	+	–	+	–	+	–	–
HA	+++	–	+	–	+	–	–	+	+	–
EI24%	+++	+	+	–	+	–	+	–	+	+
PA	+++	+	+	+	+	+	+	+	+	+
BAP	+++	–	–	–	–	–	–	–	–	–
AWD	+++	–	–	–	–	–	–	–	–	–
TAH	+++	–	–	–	–	–	–	–	–	–
BATH	+++	–	+	–	–	+	–	–	–	–
MDC	+++	–	+	–	–	+	–	–	–	–
TGS	+++	–	+	+	–	+	–	–	–	–
EU	+++	+	–	+	+	+	–	+	+	+
QM	+++	+	+	+	+	+	+	+	–	–
PM test	+++	–	–	–	–	–	–	–	–	–

OSM: Oil spreading method, **HA:**Hemolytic activity, **EI24%**: Emulsification Index, **PA:**Penetration assay, **BAP:**Blue Agar Plate, **AWD:**Anonic Well diffusion, **TAH:**Tolerance Against Hydrocarbon, **BATH:**Bacterial Adhesion to Hydrocarbon Assay, **MDC:**Modified Drop collapse, **TGS:**Tilted Glass Slide, **EU:**Emulsification Asay, **QM:**Qulalitative method, **PM test**: Parafilm M Test "+++" Very Good activity; "++" Good activity; "+" partial activity; "– "No activity

The oil spreading method is rapid and easy to carry out, requires no specialized equipment, and only requires a small volume of sample [76]. In our study, only isolates ALIG01 isolate was found to be positive by oil-spreading method indication production of biosurfactant as seen in Fig.2c. Displacement of oil clearly is a sign of extracellular surfactants present in the supernatant of cultures.

In the present investigation the penetration assay, Blue agar plate, Agar well diffusion method, tolerance against hydrocarbon, BATH assay, drop collapse test, tilted glass test, emulsification assay quantiative microplate analysis and paraflim M test with crude oil were also done to confirm biosurfactant production.

Hemolytic activity appears to be a good screening criterion in the search for biosurfactant-producing bacteria [75]. During our study, positive hemolytic activity was showed by only ALIG01 isolate with complete hemolytic activity while the other isolates ALIG02–10 showed partial hemolytic activity in Fig.3d. This test is generally carried out as a primary method for screening of biosurfactant-producing bacteria. Isolates were tested for haemolytic activity, which is regarded by some authors as indicative for biosurfactant production and used as a preliminary method for bacterial screening that was in agreement with previous findings [7, 11, 35, 80, 81, 85]. To determine the potential of a good biosurfactants Emulsification activity is one of the good criteria [74]. In the present investigation ALIG02, 03, 05, 07, 09, 10 isolates showed partial emulsification potential, and only ALIG01 isolate gave a good emulsification index > 50%) with all 3 hydrocarbons tested, which included hexane, xylene, and oil (petrol). Biosurfactants are known to have good penetration ability [71]. Our study shows that isolates producing biosurfactant display good emulsification with oil (petrol). The colored culture supernatant placed on the surface of the hydrophobic paste, resulted in color change in upper phase from red to cloudy white with ALIG01 isolates which was noted as positive test. Partial change in color or appearance of red color wells was observed in rest ALIG02–10 isolates in Fig.4.

A semi quantitative assay for the detection of extracellular glycolipids or other anionic surfactants is determined by CATB agar plate method which also known as Blue agar plate method that is based on the formation of an insoluble ion pair of anionic surfactants with the cationic surfactant CTAB and the basic dye methylene blue [40]. A dark blue halo zone was observed around ALIG01 isolate while no zone was observed in negative control and rest other isolates Fig.3e. White precipitation line between sample well and cationic compounds CTAB with barium chloride formed in only isolate ALIG01 in Fig.5. The biosurfactant derived from ALIG01 isolate (suspected Pseudomonas spp.) was confirmed as an anionic biosurfactant. Thus, under the prevailing experimental conditions, this very simple test made it possible to demonstrate the anionic character of the surfactant produced by biosurfactants producing strain. Generally biosurfactant produced from other labs were found as anionic surfactants. Xylolipid produced by *Lactococcus lactis* was also reported anionic in nature [70] which is in favour of our screening methodology.

A good characteristic feature of biosurfactant-producing bacteria is its ability to adhere to hydrocarbons. Rosenberg et al. [49] developed the bacterial adhesion to hydrocarbons method, a simple photometrical assay for measuring the hydrophobicity of bacteria. ALIG01 showed maximum adhesion to hexane while rest other isolates didn't show any adhesion. It is also possible to detect biosurfactant-producing and hydrocarbon-degrading activity simultaneously on agar plates by overlaying with hydrocarbon [72]. The hydrocarbon overlay agar method showed halos around the colony for ALIG 01 isolate. The assay was developed by Siegmund and Wagner [56] and in the present study revealed that only ALIG 01 isolate as positive as seen in Fig.3f.

The BATH assay was further confirmed by visualization of cells adhered to crude oil confirmed the affinity of cells towards crude oil facilitated by producing biosurfactant in isolate ALIG01 only which in agreement with previous findings by Kumar et al [79]. Both the drop-collapse and visualization of cells adhered to crude oil have several advantages in requiring a small volume of samples, are rapid and easy to carry out, and do not require specialized equipment.

The tilted glass slide test, developed by Persson and Molin, [47] was positive for four isolates (ALIG01, 03, 04 and 06). Water flowed over the slides on which these cultures were tested our findings are also in favour with Zhang et al. [80].

The drop-collapse test was positive in only ALIG 01, 03 and 06 isolates as soon in Fig.5b. Drop collapse result was in accordance with the work of Tambekar and Gadakh, [58]. Drop collapse test was suggested to be sensitive and easy method to test production of biosurfactant, however, Said et al. [57] have reported that microorganisms that recorded negative drop collapse test are not good emulsifiers and are not good biosurfactant producers; hence rest of the isolates were not selected for further assay. In liquid that contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension.

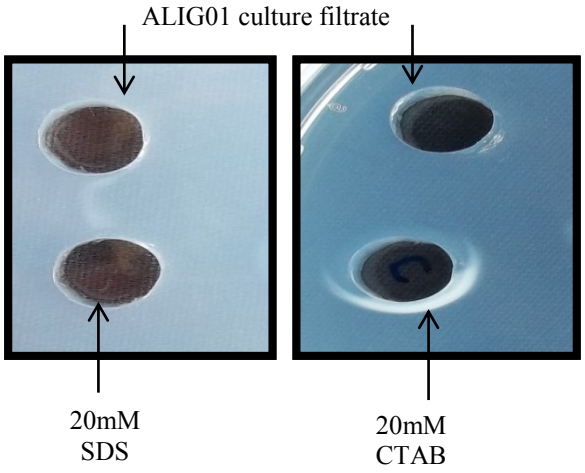


Figure 5. Double diffusion on agar of an anionic biosurfactant produced by isolate ALIG01 vs CTAB and SDS

Emulsification assay is an indirect method used to screen biosurfactant production. The assumption is that if the cell-free culture broth used in this assay contains biosurfactant, then it will emulsify the hydrocarbons present in the test solution. Emulsification assay was performed with 3 hydrocarbons and the best average was found for xylene; by examining emulsification units, it is possible to select a potent biosurfactant/bioemulsifier producer [73]. The supernatant of ALIG01 isolate was tested for surfactant activity using the qualitative microplate assay and surfactant activity could be easily detected by optical distortion of grids compared with a control as shown in Fig. 5a. Compared to the emulsification test, the microplate assay proved to be sensitive, rapid and easy to perform and could be used without specialized equipment or chemicals. This assay offers the potential for conversion to high throughput screening for biosurfactant-producing microbes before undertaking extensive experiments in biosurfactant production. The parafilm M test was also used a preliminary screening test for biosurfactant production. In our study showed the droplet diameter of culture

filtrate of ALIG01 isolate tested was larger (> 1.8cm) than incomparison with other isolates. These screening methods employed in the present study are amongst the most common methods used for confirmation of biosurfactant production, all of which are principally based on the physical effects of the biosurfactants produced.

These different assays offer the potential for conversion to high throughput screening for biosurfactant-producing microbes before undertaking extensive experiments in biosurfactant production. The presence of biosurfactants produced by microbes is easy and fast to investigate by visual inspection. Soil sample from an Automobile repairing workshop is a good source for screening of biosurfactant-producing microbes instead of going more far oil split sites locations. The bacterial isolate ALIG01 displayed the highest activity after screening with methods. Molecular identification and sequencing for this strain will be further investigated. It is important to note that most of the researchers have used maximum two or three screening methods for selection of biosurfactant producers. We suggest a single method is not suitable to identify all type of biosurfactants. Therefore, a combination of various methods is required for effective screening. To the best of our knowledge, this is the first report assessing thirteen different screening methods for selecting biosurfactant producing bacteria from an automobile workshop in Aligarh.

Acknowledgements

The authors are very thankful to Dept. of Science and Technology, Govt. of India, for providing the financial grants received under **File No. DST-SERB-NPDF/2016/001409**and Dept. of Botany, Aligarh Muslim University, Aligarh, U.P for proving all the support.

REFERENCES

1 Alloway. B J. Heavy metals in soils, 2nd Ed. Chapman and Hall, India: Australia. 1995.

2 Abidemi. O.O. Levels of Pb, Fe, Cd and Co in Soils of Automobile Workshop in Osun State, Nigeria. J. Appl. Sci. Environ. Manage. 2011. no. 15 (2). pp. 279–282.

3 Abouseoud. M, Maachi. R, Amrane. A, Boudergua. S and Nabi. A. Evaluation of different carbon and nitrogen sources in production of biosurfactant by Pseudomonas fluorescens. Desalination no. 223. 2013. pp. 143.

4 Azka A, Hareem M, S.N. Chaudhary and F.W. Comparative Analysis of Biosurfactant Production Assays by Five Indigenous Oil Sludge Bacteria. Biologia, Pakistan. no. 63(2). 2017. pp. 205–209.

5 Ali S.R, Chowdhury B.R, Mondal. P, Rajak S. Screening and characterization of biosurfactants producing microorganism form natural environment (whey spilled soil). J Nat Sci Res. no. 3(13). 2013. pp. 34 – 64.

6 Banat I.M, Franzetti A, Gandolfi I, Bestetti G, Martinotti M.G, Fracchia L. Microbial biosurfactants production, applications and future potential. Appl Microbiol Biotechnol. no. 87. 2010. pp. 427–444.

7 Banat, I.M, Makkar, R.S. Cameotra, S.S. Potential commercial Application of Microbial Surfactants. Applied Microbiol. Biotechnol. no. 53. 2000. pp. 495–508.

8 Bodour A A and Maier R.M. Application of a modified dropcollapse technique for surfactant quantification and screening of biosurfactant – producing microorganisms. J Microbiol Methods. no. 32. 1998. pp. 273–280.

9 Banat, I.M. Biosurfactants: production and possible uses in microbial enhanced oil-recovery and oil pollution remediation-a review. Bioresour. Technol. no. 51. 1995. pp. 1–12.

10 Cameotra S.S and Makkar, R.S. Recent applications of biosurfactants as biological and immunological molecules. Curr. Opin. Microbiol. no. 7. 2004. pp. 262–266.

11 Carrillo P, Mardaraz, C, Pitta-Alvarez, I.S, Giulietti, A.M. Isolation and selection of biosurfactant-producing bacteria. World Journal of Microbiology and Biotechnology. no. 12. 1996. pp. 82–84.

12 Das P, Mukherjee S, Sivapathasekaran C, Sen R. Microbial surfactants of marine origin: Potentials and prospects. Adv Exp Med Biol. no. 672. 2010. pp. 88–101.

13 Das P, Mukherjee, S, and Sen, R. Antiadhesive action of a marine microbial surfactant. Colloids Surf. B Biointerfaces. no.71. 2009a. pp. 183–186.

14 Das, P, Mukherjee, S and Sen R. Biosurfactant of marine origin exhibiting heavy metal remediation properties. Bioresour. Technol. no. 100. 2009b. pp. 4887–4890.

15 Duguid J.P. Staphylococcus: cluster-forming gram-positive cocci. In: Collee JG, Duguid JP, Fraser AG, Marmion BP, editors. Practical Medical Microbiology. 13th ed. Edinburgh, UK: Churchill Livingstone. 1989. pp. 303–316.

16 Davis, D.A, Lynch, H.C, Varley, J. The production of surfactin in batch culture by *Bacillus subtilis* ATCC 21332 is strongly influenced by the conditions of nitrogen metabolism. Enz. Micro Technol. no. 25(3–5). 1999. pp. 322–329.

17 EPA – office of air quality, planning and standards. Compilation of Air Pollutant Emission Factors AP-42, Stationary Point and Area Sources, Washington, D.C., 5 Ed., EPA. 2015. vol. 1.

18 Evans, LJ. Chemistry of metal retention by soils. Environ. Sci. Tech. no. 23(9). 1989. pp. 1046–1056.

19 Francy, D.S, Thomas, J.M, Raymond, R.L, Ward, C.H. Emulsification of hydrocarbon by surface bacteria. J Industrial Microbiol, vol. 8. 1991. pp. 237–46.

20 Gautam, K.K and Tyagi, V.K. Microbial surfactants: a review. J. Oleo Sci. no.55. 2006. pp. 155–166.

21 Georgiou, G, Lin, S.C and Sharma, M.M. Surface active compounds from microorganisms. Biotechnology, no. 10. 1990. pp. 60–65.

22 Hewald, S, Josephs, K and Bölker, M. Genetic Analysis of Biosurfactant Production in Ustilago maydis, Applied and Environmental Microbiology, no. 71(6). 2005. pp. 3033–3040.

23 Ibrahim M. Banat, Andrea Franzetti, Isabella Gandolfi, Giuseppina Bestetti, Maria G. Martinotti, Letizia Fracchia, Thomas J. Smyth and Roger Marchant. Microbial biosurfactants production, applications and future potential. Appl Microbiol Biotechnol. no. 87. 2010. pp. 427–444.

24 Jain D. K, Collins-Thompson D.L, Lee H.A drop collapsing test for screening surfactant-producing microorganisms. J Microbiol Methods, no. 13. 1991. pp. 271–279

25 Koglin A, Doetsch V and Bernhard F. Molecular engineering aspects for the production of new and modified biosurfactants. Adv Exp Med Biol., no. 672. 2010. pp. 158–169.

26 Lima T.M, Procópio L.C, Brandão F.D, Carvalho A.M, Tótola M.R. and Borges A.C. Biodegradability of bacterial surfactants. Biodegradation, no. 22. 2011. pp. 585–592.

27 Lourith N, Kanlayavattanakul M. Natural surfactants used in cosmetics: Glycolipids. Int J Cosmet Sci. no. 31. 2009. pp. 255 – 261.

28 Martinez A, Ostrovsky P, Nunn D N. Identification of an additional member of the secretin superfamily of proteins in Pseudomonas aeruginosathat is able to function in type II protein secretion. Mol Microbiol no. 28. 1998. pp. 1235–1246.

29 Meylheuc T, Van Oss C.J, Bellon, Fontaine MN. Adsorption of biosurfactant on solid surfaces and consequences regarding the bio-adhesion of Listeria monocytogenes LO28. J Appl Microbio. no. 91(5). 2001. pp. 822–832.

30 Miller, J.H. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1972. pp. 433.

31 Morita, T, Konishi, M, Fukuoka, T, Imura, T. and Kitamoto, D. Physiological Differences in the Formation of the Glycolipid Biosurfacants, Mannosylerythritol Lipids, between Pseudozyma antarticaand Pseudozyma aphidis, Applied of Microbiology and Biotechnology, no. 74. 2007. pp. 307–315.

32 Morikawa M, Diado H, Takao T, Murata S, Shimonishi Y, Imanaka T.A new lipopeptide biosurfactant produced by Arthrobacter sp. strain MIS38. J Bacterio. no. 175. 1993. pp. 6459–6466.

33 Morikawa M, Hirata Y, Imanaka T. A study on the structure function relationship of lipopeptide biosurfactants. Biochim Biophys Acta. no. 1488. 2000. pp. 211–218.

34 Mukherjee, S, Das, P and Sen, R. Towards commercial production of microbial surfactants. Trends Biotechnol. no. 24. 2006. pp. 509–515.

35 Mulligan, C.N. Cooper, D.G. Neufeld, R.J. Selection of microbes producing biosurfactants in media without hydrocarbons. J. Ferment. Technol. no. 62. 1984. pp. 311–314.

36 Ng L.S, Tan T.Y, Yeow, S.C.A cost-effective method for the presumptive identification of Enterobacteriaceae for diagnostic microbiology laboratories. Pathology. no. 42. 2010. pp. 280–283.

37 Nguyen, T. T, Youssef, N. H, McInerney, M.J. and Sabatinic, D.A. Rhamnolipid biosurfactant mixtures for environmental remediation. Water Res. no. 42. 2008. pp. 1735.

38 Nitschke M, Costa S. Biosurfactants in food industry. Trends Food Sci Technol. no. 18. 2007. pp. 252–259.

39 Pelczar M.J Jr, Reid R.D.1958. Microbiology. New York, NY, USA: McGraw-Hill.

40 Pinzon, N.M, Ju, L.K. Improved detection of rhamnolipid production using agar plates containing methylene blue and cetyl trimethylammonium bromide. Biotechnology Letters, no. 31(10). 2009. pp. 1583–1588.

41 Persson, A. and Molin, G. Capacity for biosurfactant production of environmental Pseudomonas and Vibrionaceae growing on carbohydrates. Appl. Microbiol. Biotechnol. no. 26. 1987. pp. 439–442.

42 Pruthi, V. and Cameotra, S.S. Effect of nutrients on optimal production of biosurfactants by Pseudomonas putida – a Gujarat oil field isolate. J. Surfact. Deterg. no. 6. 2003. pp. 65.

43 Pirôllo, M.P. S, Mariano, A. P, Lovaglio, R. B, Costa, S.G.V.A. O, Walter, V, Hausmann, R. and Contiero, J. Biosurfactant synthesis by Pseudomonas aeruginosaLBI isolated from a hydrocarbon – contaminated site. J. Appl. Microbiol. no. 105. 2008. pp. 1484.

44 Providenti, M. A, Flemming, C. A, Lee, H. and Trevore, J.T. Effect of addition of rhamnolipid biosurfactants or rhamnolipid producing Pseudomonas aeruginosaon phenanthrene mineralization in soil slurries. FEMS Microbiol. Ecol. 1995. no. 17. pp. 15.

45 Poremba, K, Gunkel, W, Lang, S and Wagner, F. Marine biosurfactants, III. Toxicity testing with marine microorganisms and comparison with synthetic surfactants. Z. Naturforsch. no. 46. 1991. pp. 210–216.

46 Rahman, K. S, Banat, I. M, Thahira, J, Thayumanavan, T. and Lakshmanaperumalsamy, P. Bioremediation of gasoline contaminated soil by a bacterial consortium amended with poultry litter, coir pith and rhamnolipid biosurfactant. Bioresour. Technol. no. 81. 2002a. pp. 25.

47 Robert, M, Mercade, E, Bosch, M. P, Parra, J. L, Espuny, M. J, Manresa, M.A. and Guinea, J. Effect of the carbon source on biosurfactant production by Pseudomonas aeruginosa 44Ti. Biotechnol. Lett. no. 11. 1989. pp. 871.

48 Rosenberg E, Zuckerberg A, Rubinovitz C, Gutnick DL. Emulsifier of *Arthrobacter*RAG-I: isolation and emulsifying properties. Appl Environ Microbiol. no. 37. 1979. pp. 402–408.

49 Rosenberg M, Gutnick D.L, Rosenberg E. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol Lett. no. 9. 1980. pp. 29–33

50 Rodrigues L.R, Teixeira J.A, Van Der Mei H.C, Oliveira R. Physicochemical and functional characterization of a biosurfactant produced by Lactococcus lactis. Colloids Surf B Biointerfaces, no. 49. 2006. pp. 79–86.

51 Rodrigues, L, Banat, I. M, Teixeira, Jand Oliveira, R. Biosurfactants: potential applications in medicine. J. Antimicrob. Chemother, no.57. 2006. pp. 609–618.

52 Satpute, S. K, Bhawsar, B.D, Dhakephalkar P K, Chopade, B.A.2008. Assessment of different screening methods for selecting biosurfactant producing marine bacteria. Indian J Marine Sci. no. 37. 2008. pp. 243–250.

53 Satpute, S.K, A.G. Banpurkar, P.K. Dhakephalkar, I.M. Banat and B.A. Chopade. Methods for investigating biosurfactants and bioemulsifiers: A review. Crit. Rev. Biotechnol. no. 30. 2010. pp. 127–144.

54 Singh, P and Cameotra, S.S. Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol. no. 22. 2004. pp. 142–146.

55 Shoeb, E. Genetic Basis of Heavy Metal Tolerance in Bacteria. Ph. D. thesis. University of Karachi, Karachi, Pakistan, 2006.

56 Siegmund I, and Wagner F. New method for detecting rhamnolipids excreted by *Pseudomonas*species during growth on mineral agar. Biotechnol Tech., no. 5. 1991. pp. 265–268.

57 Said BH, Latifa S, Abdeljelil G, Abderrazak M. Screening of potential biosurfactant producing bacteria isolated from seawater biofilm. Afri. J. Biotech.11. no. 77. 2012. pp. 14153–14158.

58 Tambekar D. H, and Gadakh P.V. Biochemical and molecular dectection of biosurfactant producing bacteria from soil. Int. J. Life Sci. Biotech. & Pharma Res., no. 2(1). 2013. pp. 204 – 211.

59 Tomar S, Singh B.P, Lal M, Khan M.A, Hussain T, Sharma S, Kaushik S.K, Kumar S. Screening of noval microorganism for biosurfactant and biocontrol activity against. Phytophthora infestans. J Env Biol. no. 35. 2014. pp. 893–899.

60 Usman, M.M, Dadrasnia, A, Tzin Lim, K, Mahmud, A.F, Ismail, S. Application of biosurfactants in environmental biotechnology; remediation of oil and heavy metal. AIMS Bioengineering, no. 3(3). 2016. pp. 289–304.

61 Vijaya kumar S. and Saravanan V. Biosurfactant stypes, sources and applications. Res. J. Microbiol. no. 10. 2015. pp. 181 – 192.

62 Vaux, D, Cottingham, M. Method and apparatus for measuring surface configuration, patent number GB0001568.5. 2001.

63 Walter V, Syldatk C, Hausmann R. Screening concepts for the isolation of biosurfactant producing microorganisms. Adv Exp Med Biol. 2010. no. 672. pp. 1–13.

64 Walter, V, C. Syldatk and R. Hausmann. Screening Concepts for the Isolation of Biosurfactant Producing Microorganisms. In: Biosurfactants, Ramkrishna, S. (Ed.). vol. 672. Landes Bioscience and Springer Science, New York, 2010 ISBN: 9781441959799.

65 Xu Q, Nakajima M, Liu Z. and Shiina T. Biosurfactants for microbubble preparation and application. Int J Mol Sci. no. 12. 2011. pp. 462–475.

66 Urum, K, Pekdemir, T. Evaluation of biosurfactants for crude oil contaminated soil washing. Chemosphere, no. 57. 2004. pp. 1139–1150.

67 Yen Chen C, Baker S.C, Richard C. Darton R.C. The application of a high throughput analysis method for the screening of potential biosurfactants from natural sources. Journal of Microbiological Methods. no. 70. 2007. pp. 503–510.

68 Zhang, G. L, Wu, Y. T, Qian, X.P. and Meng, Q. Biodegradation of crude oil by *Pseudomonas aeruginosa*in the presence of rhamnolipids. J. Zhejiang Univ. Sci. B, no. 6. 2005. pp. 725.

69 Zajic, J.E, Gignard, H and Gerson, D F. Properties and biodegradation of a bioemulsifier from Corynebacterium hydrocarboclastus. Biotechnol. Bioeng. no. 19. 1977. pp. 1303–1320.

70 Saravanakumari P, and Mani K. Structural characterization of a novel xylolipid biosurfactant from Lactococcus lactis and analysis of antibacterial activity against multi-drug resistant pathogens. Bioresource Technol. no. 101(22) 2010. pp. 8851–8854.

71 Youssef N.H, Duncan K.E, Nagle D.P, Savage K.N, Knapp R.M, McInerney M.J.J. Comparison of methods to detect biosurfactant production by diverse microorganisms. Microbiol Methods. no. 56(3). 2004. pp. 339–347.

72 Kokare, C.R, Kadam, S.S, Mahadik, K.R, Chopade, B.A. Studies on bioemulsifier production from marine Streptomyces sp. S1. Indian J Biotechnol. no. 6. 2007. pp. 78–84.

73 Belcher, R.W, Huynh, K.V, Hoang, T.V, Crowley, D.E. Isolation of biosurfactant-producing bacteria from the Rancho La Brea Tar Pits. World J Microbiol Biotechnol. no. 28. 2012. pp. 3261–3267.

74 Bonilla, M, Olivaro, C, Caronal, M, Vazquez, A, Soubes, M. Production and characterization of a new bioemulsifier from Pseudomonas putida ML2. J Appl Microbiol. no. 98. 2005. pp. 456–463.

75 Carter, G. Diagnostic Procedures in Veterinary Bacteriology and Mycology. 4th ed. Springfield, IL, USA: Charles C Thomas. 1984.

76 Plaza, G, Zjawiony, I, Banat, I. Use of different methods for detection of thermophilic biosurfactant-producing bacteria from hydrocarbon-contaminated bioremediated soils. J Petro Science Eng. no. 50. 2006. pp. 71–77.

77 Shoeb E, Badar U, Akhter J, Shams H, Sultana M, Ansari M.A. Horizontal gene transfer of stress resistance genes through plasmid transport. World J Microbiol Biotechnol. no. 28. 2012. pp. 1021–1025.

78 Moran A, Alejandra M, Martinez F, et al. Quantification of surfactin in culture supernatant by hemolytic activity. Biotechnol Lett. no. 24. 2002. pp. 177–180.

79 Kumar, PA, Janardhan A, Viswanath B, Monika, K, Jung, Y.J, Narasimha, G. Evaluation of orange peel for biosurfactant production by Bacillus licheniformis and their ability to degrade naphthalene and crude oil. 3 Biotech. 2016. no. 6. pp. 43.

80 Zhang, J., Xue, Q., Gao, H., Lai, H., Wang, P.Bacterial degradation of crude oil using solid formulations of bacillus strains isolated from oil-contaminated soil towards microbial enhanced oil recovery application. RSC Advances, no. 6 (7). 2016. pp. 5566–5574.

81 Zhang, X., Xu, D., Zhu, C., Lundaa, T., Scherr, K.E. Isolation and identification of biosurfactant producing and crude oil degrading Pseudomonas aeruginosa strains. Chemical Engineering Journal. no. 209. 2012. pp. 138–146.

82 Sakthipriya, N., Doble, M., Sangwai, J.S. Action of biosurfactant producing thermophilic Bacillus subtilis on waxy crude oil and long chain paraffins. International Biodeterioration and Biodegradation. no. 105. 2015. pp. 168–177.

INFORMATION ABOUT AUTHORS

Touseef Hussain Plant Pathology Section, Environmental Lab, Dept. of Botany, Aligarh Muslim University, Aligarh 202002, U.P, India, Hussaintouseef@yahoo.co.in

Abrar Ahmad Khan Plant Pathology Section, Environmental Lab,Dept. of Botany, Aligarh Muslim University, Aligarh 202002, U.P, India, Khanab2009@gmail.com

CONTRIBUTION

Touseef Hussain proposed a scheme of the experiment, organized production trials, wrote the manuscript, correct it before filing in editing and is responsible for plagiarism.

Abrar Ahmad Khan guided while during the research study

CONFLICT OF INTEREST

The authors declare no conflict of interest.

RECEIVED 7.17.2018

ACCEPTED 8.30.2017