

Screening and Comparative Analysis of Biosurfactant-producing Bacteria Isolated from Variably Contaminated Sites along Karachi Coast

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KEYWORDS

ABSTRACT

Biosurfactant
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The object of this study was to screen and compare biosurfactant-producing bacteria isolated from variably contaminated sampling sites of the Arabian Sea coast of Karachi. Sampling site was divided into three categories based on contamination: “highly contaminated (HC)”, “moderately contaminated (MC)”, and “undisturbed contaminated (UC)”. The isolates were screened through a combination of seven screening tests. All tests were performed using three hydrocarbons: crude oil, xylene, and hexane. For hemolytic activity, 36% of isolates from MC sites showed the highest activity. The results of the oil spreading method revealed the highest percentage (40%) of the isolates from the UC area. The Bacterial Adhesion to Hydrocarbon (BATH) assay, emulsification index, and emulsification assay revealed positive adherence and emulsification with at least one of the hydrocarbons tested in more than 95% of the isolates, regardless of the sampling area. The highest adherence was observed with hexane in the maximum number of isolates. The maximum emulsification index was recorded in isolates with crude oil. In the emulsification assay, the best results were observed with xylene. For the Cetyltrimethyl ammonium bromide (CTAB) agar plate assay and drop-collapse method, the highest percentages of isolates (60% and 96%, respectively) from the HC area showed positive results. These results indicate the potential of biosurfactant production in isolates, but performance varied with respect to different screening tests and hydrocarbons tested. Biosurfactants are naturally occurring amphiphilic molecules present in the environment. They are efficiently used in various industries such as food processing, the pharmaceutical industry, healthcare, agriculture, and in the bioremediation of polluted environments. The isolation of microbes from marine water is a potential source of biosurfactants. Biosurfactants offer advantages such as low toxicity, easy biodegradability, high foaming properties, and the ability to perform under extreme conditions. These molecules can reduce surface and interfacial tension in both aqueous and hydrophobic phases. As compared to chemically synthesized surfactants, microbial-produced surfactants are miscellaneous and structurally diverse in nature. These distinctive properties of biosurfactants make these a potential user for industrial applications and the best alternative to synthetic surfactants.

1. Introduction

The Karachi coast receives 472,000m³ of domestic and industrial wastewater per day, which is the main cause of increasing levels of contamination level in this area (Monawwar *et al.*, 2005). In addition, hydrocarbons contaminate harbor sediments from shipping activities, fuel oil spills, and runoffs, which are becoming a serious environmental issue since hydrocarbons are toxic to all biological systems (Olivera *et al.*, 2003). When oil spreads in the environment, low-molecular-weight hydrocarbons are volatilized while polar components are dissolved in water. However,

most of the oil hydrocarbons remain on the water surface or adhere to soil particles due to their low solubility (Brinda *et al.*, 2023). Biosurfactant-producing bacteria are often found in hydrocarbon-contaminated areas. Biosurfactants are microbial surface-active agents produced by certain microorganisms during their growth phase, and may be extracellular or intracellular in nature (Kumar *et al.*, 2021). Surfactants derived from microbial sources represent a broad spectrum of biomolecules that emulsify oil-water mixtures and have potential application for bioremediation (Zhang *et al.*, 2012). These

amphiphilic compounds include fatty acids, glycolipids, polysaccharide lipid complex, lipoprotein, or phospholipids.

Bacterial surface-active compounds have similar properties to chemically produced surfactants, but they are less toxic, biodegradable, and can be produced in situ, at the contaminated site (Cha, 2000). Hydrocarbon-degrading bacteria release surfactants/emulsifiers to facilitate assimilation of these insoluble substrates (Obayori *et al.*, 2022). Bacteria capable of emulsifying and solubilizing hydrophobic contaminants in situ may have a distinct advantage over competitors in contaminated areas, and therefore, samples from such sites are often rich in biosurfactant producing bacteria (Al-Marri *et al.*, 2023).

Efficiency of biological sources for the treatment of hydrocarbon-based pollutants is based on biodegradability contrary to the chemical-based treatment. Biosurfactant producing bacteria can play an important role in the safe and swift bioremediation of hydrocarbon contaminated sites. These bacteria can also be used in enhanced oil recovery and may be considered for other potential applications in environmental protection. Biosurfactants have several advantages over the chemical surfactants such as lower toxicity, higher biodegradability, better environmental compatibility, potentially high activities, and stability at extremes of temperature, pH, and salinity (Srivastava *et al.*, 2022). Demand of biosurfactant is increasing day by day and they are getting much attention as they represent environmentally friendly, “green” chemicals. The range of industrial applications of biosurfactants include enhanced oil recovery, crude oil drilling, lubricants, and bioremediation of pollutants, health care and food processing (Karnwal *et al.*, 2023).

2. Materials and Methods

2.1 Sampling Area

For the isolation of biosurfactant producing

bacteria, seawater samples were collected from coastal areas of Karachi. Sampling areas were categorized as highly contaminated (HC), moderately contaminated (MC) and undisturbed/ uncontaminated (UC) because of visible contamination and anthropogenic activities in different areas. HC area was considered near the harbor, oil terminal and dockyard; MC in the open sea, marine check and Manora; and UC was near the Oyster Rock Island which is undisturbed area of the Sea (Figure and Table 1). A total of 14 sampling sites were selected: 5 from HC and MC each and 4 from UC area. For the isolation of bacterial isolates marine water samples were collected in sterilized glass tubes and kept at 4°C for further use.



Figure 1: Some sampling sites at Karachi coast

2.2 Enrichment and Isolation of Bacterial Isolates

Seawater samples were serially diluted and spread on R2A (Reasoner's 2A agar) (Anandaraj and Thivakaran, 2010) agar plates, incubated at 37°C for 24 h. After incubation, plates were enumerated, and morphologically distinct bacterial colonies were selected (approximately 5 to 6 colonies per plate) and purified by re-streaking twice on Luria Bertani (LB) agar plate to obtain pure cultures. The selected bacterial isolates were stored in LB agar slants and kept under refrigerated conditions for further screening (Shoeb, 2006).

2.3 Identification and characterization of isolated strains

2.3.1 Cellular morphology

Cells were observed with Gram staining under a microscope (oil immersion, 100×). The shape of the cells (cocci, bacilli, and coccobacilli) and arrangement of cells (scattered, bunches, and chains) along with the Gram reaction were observed (Shoeb *et al.*, 2015).

2.4 Screening Methods for Biosurfactant Production

2.4.1 Culture medium and bacterial growth

One loop of each bacterial strain was transferred to a test tube containing 10 mL of LB broth. Culture tubes were maintained in a shaker for 24 hs at 37°C. After incubation, the culture broth from each tube was centrifuged at 8000 rpm for 15 min (Varadavenkatesan and Murty, 2013). The cell-free supernatant obtained after centrifugation was used for the dropping collapse test, oil spreading assay, emulsification index, and emulsification assay (EU/mL). The bacterial cells were used for hemolytic assay, BATH assay, and CTAB agar plate method.

2.4.2 Hemolytic activity

Hemolytic assay was performed in blood agar plates (Thavasi *et al.*, 2011). Broth (50 µL) cultures were spot inoculated onto blood agar plates and incubated for 48 h at 37°C. The plates were visually inspected for a zone of clearance (hemolysis) around the colony. The diameter of the zone of clearance is a qualitative method used as an indicator of biosurfactant production.

2.4.3 Oil Spreading Method

The oil spreading technique was carried out according to the method described by Youssef *et al.*, 2004 and Plaza *et al.*, 2006. 50 mL of distilled water was added to the Petri dishes, followed by the addition of 100µL of crude oil to the surface of the water. Then 10 µL of cell-free

culture broth was dropped onto the crude oil surface. The occurrence of a clear zone was an indication of biosurfactant production. The diameter of the clear zone on the oil surface was measured and compared to 10 µL of distilled water as a negative control.

2.4.4 CTAB Agar Plate

Isolated bacterial strains were cultured on light blue mineral salts agar plate containing the Cetyl tri-methyl ammonium bromide (0.2 mg/mL) and the basic dye methylene blue (5 mg/mL). CTAB agar plates were used to detect extracellular glycolipid production (Siegmond and Wagner, 1991; Fenibo *et al.*, 2019). Biosurfactants were observed by the formation of dark blue halo around the colonies.

2.4.5 Drop-collapse test

Screening of biosurfactant production was performed using the qualitative drop-collapse test described by Jain *et al.* 1991 and Mohanram *et al.*, 2016). 2 µL crude oil was applied to well regions delimited on the covers of 96-well micro plates, left to equilibrate for 24 h. Five microliters of the 48h cell-free culture broth, after centrifugation at 12,000g for 5 min to remove cells, was transferred to the oil-coated well regions, and the drop size was observed 1 min later. The drop diameter was compared with deionized water as a negative control.

2.4.6 Bacterial adhesion to hydrocarbons (BATH) Assay

BATH assay was performed as previously described by Rosenberg *et al.* 1980. Bacterial cells were washed twice and suspended in a buffer salt solution (g/L 16.9 K₂HPO₄, 7.3 KH₂PO₄) to give an optical density (OD) at 600 nm of ~ 0.5. The cell suspension (2 mL) with 100µL crude oil added was vortexed and shaken for 3 min in test tubes. After shaking, crude oil and aqueous phase were allowed to separate for 1 h. Absorbance of the aqueous phase was

measured again at 600nm (Thavasi *et al.*, 2011). Hydrophobicity is expressed as the percentage of cell adherence to crude oil, calculated as follows:

$$\text{Percentage of bacterial cell adherence} = 1 - \frac{\text{OD of Aqueous phase}}{\text{OD of initial cell suspension}} * 100$$

2.4.7 Emulsification Index (E24)

The emulsification index (E24) was measured using the method described by Ilori *et al.* (2005) to check the stability of the biosurfactant extracted. Emulsification index was measured by adding 2 mL of crude oil to 2 mL of cell-free extract and vortexed at high speed for 2 min. Measurement was taken after 24 h. Emulsions formed by the isolates were compared to those formed by a 1% (w/v) solution of the synthetic surfactant, Sodium Dodecyl Sulphate (SDS) in deionized water (Chooklin *et al.*, 2023). The emulsification activity was determined using the following formula:

$$\text{Emulsification Index} = \left(\frac{\text{Height of emulsion layer}}{\text{Height of liquid column}} \right) \times 100$$

2.4.8 Emulsification assay

Cell-free culture broth was used as the biosurfactant source to check the emulsification of crude oil, xylene, and hexane. 1 mL of cell-free culture broth was added to 5 mL of 50 mM Tris buffer (pH 8.0) in a 30 mL screw-capped test tube. Five milligrams of hydrocarbon were added to the above solution, vortex-shaken for 1 min, and the emulsion mixture was allowed to stand for 20 min. OD of the emulsion mixture was measured at 610 nm (Dharmadevi *et al.*, 2022). A negative control was a buffer solution, and Triton X-100 was the positive control.

3. Results

3.1 Isolation of Bacterial isolates

A total of 89 bacterial strains were isolated, coded as DGHE01-89. 28 isolates were isolated from

heavily contaminated (HC), 22 from moderately contaminated (MC), and 39 bacterial isolates were isolated from uncontaminated areas (UC). Detailed results of morphological characterization are reported previously by Shoeb *et al.* 2015.

3.2 Identification and characterization of isolated strains

3.2.1 Cellular morphology

Cellular morphology such as arrangement, shape and Gram reaction was observed during Gram staining of isolates. The cellular shape of isolate from HC areas were short rods and round. The cellular shape of the strain was found as short rod, round, whereas cellular arrangement was found in chain and scattered form or bunches (Table 2). Results of Gram staining showed that 78%, 72% and 61% of isolates were gram negative from heavy, low, and uncontaminated areas while 21%, 27% and 38% isolates were gram positive from respective areas (Figure 2).

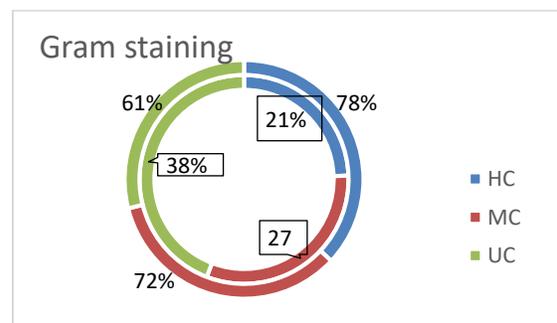


Figure 2: Gram staining of isolates

3.3 Screening for Biosurfactant Production

All the isolates were screened for their biosurfactant activity through following tests.

3.3.1 Hemolytic assay

In this study 17% of isolates from HC area showed positive hemolytic activity while 36% from MC and 30% from UC area were positive for hemolysis (Figure 3).

3.3.2 Oil Spreading Method

The oil spreading technique indicated positive oil displacing ability in 35% of the total bacterial isolates from HC area; 31% from MC; 40% isolates from UC area (Figure 3).

3.3.3 CTAB Agar Plate

In CTAB agar plate assay 60% bacterial isolates from HC produced dark blue halos around the colonies, 36% from MC and 38% from UC (Figure 3).

3.3.4 Drop collapse test

In the drop collapse test 96% of the isolates showed positive results from HC area, while 77% from MC and 79% from UC areas were positive. Results are shown in Figure (3).

3.3.5 Bath Assay

Results for BATH assay revealed that all the isolates irrespective of sampling areas were showing positive adherence to at least one of the three hydrocarbons tested. Isolates from HC area showed 32% adherence with crude oil, 39% with xylene and 60% with hexane. From the MC area 31% of bacterial strains were positive for crude oil, 40% for xylene and 45% for hexane. Similarly, isolates from UC area revealed that 43% were positive with crude oil, 58% with xylene, and 64% with hexane. As shown in figure (4), all the isolates exhibited highest cell adherence with hexane as compared to xylene and crude oil.

3.3.6 Emulsification Index (E24)

95% of all the isolates showed positive emulsification with at least one of the three hydrocarbons tested. 89% isolates from HC area showed positive emulsification with hexane, 42% with xylene and 82% with crude oil. Similarly, from MC area 63% of isolates showed positive emulsification with hexane, 45% with xylene and 68% with crude oil. Among the UC area 56% of

isolates showed positive emulsification with hexane, 46% with xylene and 79% with crude oil. Maximum emulsification index was observed in crude oil for MC and UC but for HC area isolates emulsification index was best with hexane (Figure 5).

3.3.7 Emulsification Assay

Emulsification assay was positive for all the isolates with at least one of the three hydrocarbons tested. From HC area 28% isolates with crude oil, 53% with xylene and 28% with hexane showed positive results. From MC area 40% isolates were positive with crude oil, 59% with xylene and 31% with hexane. From UC area 20% were isolated with crude oil, 23% with xylene and 28% with hexane were positive for emulsification assay. Results are shown in Figure (6).

4. Discussion

The marine environment is highly diverse, subject to extreme temperature, pressure, salinity and nutrient availability. It is a known habitat of versatile microorganisms which are capable of surviving within these types of environmental niches. Biosurfactants are amphiphilic molecules with enormous variety, environmental acceptability, wide range of functions and broad industrial applications. There has been an increased interest in the production of biosurfactant by marine microorganism particularly in the treatment of oil-spills that is why for the present study we selected Karachi Coast of Arabian Sea as our sampling site.

The tides of the Karachi Harbour are semi diurnal type due to which effluents received by the Karachi harbour are not completely flushed out into the open sea during tidal cycles (Monawwar, 2005). Considering these poor circulation conditions, we have collected water samples from different sites of coastal area and categorized them as heavily contaminated (HC), moderately

contaminated (MC) and undisturbed/ uncontaminated area (UC). Harbor, oil terminal and dockyard were all highly contaminated regions of Sea taken as HC area; Open Sea, marine check and Manora were a little distant and separated and comparatively cleaner regions were selected as MC region; and Oyster Rock Island was a totally isolated and undisturbed region was selected as UC area for sampling.

Initial culturing was done in R2a medium which is a selective medium for biosurfactant producing bacteria (Anandaraj and Thivakaran, 2010). The primary screening of biosurfactant producing bacteria was carried out through combination of seven screening tests including oil-spreading test, hemolytic activity, BATH assay, emulsification assay, emulsification index (E24), drop-collapse method and CTAB agar plate assay. The advantage of using these screening methods includes low cost, quick implementation, simple and no special equipment requirement (Walter *et al.*, 2010). All the tests were conducted using three hydrocarbons crude oil, xylene, and hexane. Crude oil is used to make petroleum products which contaminate the environment. Xylene and hexane are also major components of pollutants in our environment. Therefore, these hydrocarbons were used to estimate biosurfactant production of isolates.

Three screening tests in our study revealed almost similar results in all the three areas for BATH assay, emulsification index (E24) test and emulsification assay test. Dorobantu *et al.*, (2004) mentioned the occurrence of similar activity in almost all the biosurfactant producing isolates of these regions indicate that biosurfactant producing bacteria are capable of adherence and emulsification irrespective of the environment.

Hemolytic assay is widely used method to screen biosurfactant production. All isolates were tested for hemolytic activity and 36% of isolates from MC area showed positive activity which was

higher than HC and UC areas. It is often used as a preliminary rapid screening method for bio-surfactant producing bacterial isolates (Banat *et al.*, 2010). Many bio-products can cause red blood cell lysis having hemolytic activity are not necessarily active surface molecules. Therefore, some biosurfactant producers showed negative hemolytic activity (Youssef *et al.*, 2004).

The oil spreading method is rapid and easy to carry out as mentioned by Plaza *et al.*, 2006. It can be applied when the activity and quantity of biosurfactant is low and that is why we have got very high frequency of isolates with positive oil spreading activity from uncontaminated areas where exposure to contaminants was lower than other two areas.

In CTAB agar plate methods we have found most of the isolates with positive results from HC area. CTAB is a semi quantitative assay for detecting extra cellular glycolipids or other anionic surfactants. Microbes growing on the salt agar plate if secreted anionic surfactants produce dark blue halos. The secretion is suspected to be induced in presence of contaminants that is why higher frequency of isolates from HC gave positive results. As Rajesh *et al.*, (2017) reported that the yield of the biosurfactant greatly depends on the nutritional environment of the growing organism.

Drop collapse method was described by Jain *et al.*, (1991) according to which the drop of liquid containing a biosurfactant collapses and spreads over the oily surface, whereas the drop lacking biosurfactant remains beaded due to the hydrophobicity of the oil surface. According to Faisal *et al.*, (2023) results obtained from drop collapse assay are very accurate and consistent. In our studies 96% of the isolates from HC area showed positive results with crude oil. Degree of collapse of the culture supernatant describes the surfactant concentration, and we can suspect high concentration of surfactants in isolates recovered from HC area.

Biosurfactant producing ability in bacteria is their defense mechanism against pollutants (Amiriyan *et al.*, 2004). In our studies, isolates showed different and distinct responses when exposed to different hydrocarbons suggesting a proper selection of hydrocarbon source is necessary for determination of biosurfactant producing ability of bacteria.

5. Conclusion

In the present investigations CTAB agar plate, drop collapse test are two screening tests that showed positive activity in the areas of higher concentration. Oil spreading assay and hemolytic activity test showed better production of surfactant at lower level of contaminants. Whereas BATH assay, emulsification assay and emulsification index (E24) showed consistent results in different areas irrespective of level of pollution which showed that adherence and emulsification potential is predetermined in biosurfactant producing bacteria.

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