Screening and characterization of biosurfactant-producing bacteria isolated from the Arabian Sea coast of Karachi

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Abstract: The aim of the present study was to investigate biosurfactant-producing culturable bacteria inhabiting the coast of the Arabian Sea at Karachi. Overall, 15 seawater samples were collected from the Arabian Sea coast of Karachi. Isolation, characterization, and screening for 89 biosurfactant-producing bacterial strains were conducted through 8 conventional screening tests. Through GSP agar plate method 22 strains were found to be Pseudomonas aeruginosa and Gram reaction revealed 70% of the isolates to be gram-negative. Furthermore, 24% of the isolates showed hemolytic activity, 44% exhibited positive results for oil-spreading test, 54% showed emulsification to at least 1 of the 3 hydrocarbons tested, BATH assay results indicated maximum adhesion for hexane, 52.8% produced positive results for CTAB agar plate assay, drop-collapse activity was found in 84% of the isolates, and emulsification assay revealed highest emulsification for xylene. Findings revealed none of the isolates to be negative for every screening test conducted, while only one gram-negative isolate, DGHE65, identified as Pseudomonas aeruginosa, was positive for all the tests for biosurfactant production. Results indicate that these isolates have potential for future environmental friendly applications such as bioremediation and industrial biotechnology.

Key words: Biosurfactant, bioremediation, contamination, Arabian Sea, Karachi coast

1. Introduction

Synthetic surfactants have applications in the biodegradation processes of organic compounds, but they are environmentally hazardous. Some bacterial strains can produce active agents that are able to emulsify oil in water, assisting in biodegradation of compounds. Known as biosurfactants, they are environment friendly. These natural surfactants are amphiphilic compounds produced on living surfaces, recognized as the natural source of emulsifiers, which could be a useful instrument in the bioremediation of crude oil pollution (Karanth et al., 1999). Biosurfactants increase the solubility of hydrocarbon, which in turn increases its bioavailability to microorganisms (Zhang and Miller, 1992).

Biosurfactant-producing microbes are considered a generous gift of nature due to their diversity, environmentally friendly nature, possibility for large-scale production, selectivity, performance under extreme conditions, and potential applications in environmental protection (Banat et al., 2000). Interest in these microbes has been steadily increasing owing to the increasing need for bioremediation in our environment (Porob, 2014). Besides bioremediation, biosurfactants also have the potential to be used in enhanced oil recovery, herbicide and pesticide formulations, detergents, health care and cosmetics, pulp and paper, coal, textiles, ceramic processing and food industries, uranium ore-processing, and mechanical dewatering of peat (Shoeb, et al., 2013; Kaya et al., 2014).

Mostly bacteria and yeasts are known to synthesize biosurfactants (Ebrahimi and Tashi, 2012). These microorganisms synthesize a wide range of chemicals with surface activity, such as glycolipids and phospholipids (Rosenberg and Ron, 1999). The present study focused on the isolation, purification, screening, and characterization of biosurfactant-producing bacteria from the Karachi coast of the Arabian Sea. Considering leakage of crude oil from tankers in harbors and other anthropogenic activities, we suspected the occurrence of biosurfactant-producing bacteria at our sampling site (Ebrahimi et al., 2011). There have been very few studies so far that evaluated the presence of natural, indigenous biosurfactant-producing...
For efficient detection of potential biosurfactant producers, a combination of various screening methods is required, which were successfully evaluated in the present study.

2. Materials and methods

2.1. Sample Collection

The sampling was carried out in January 2014 and 15 seawater samples were collected from different regions of the Karachi coastal area. Samples were collected in sterile 15-mL universal glass bottles. The sampling site is shown in Figure 1.

2.2. Enrichment and isolation of bacterial isolates

Seawater samples (100 µL) were directly spread on solidified R2A medium (Oxoid) plates (Anandaraj and Thivakaran, 2010) and incubated aerobically at 37 °C for 24–48 h. Isolated colonies were picked and inoculated in Luria Bertani (LB) broth (Sambrook et al., 1989). Through successive subculturing on LB agar plates, 89 morphologically distinctive bacterial colonies were purified and kept under refrigerated conditions for further screening.

2.3. Identification and characterization of isolated strains

2.3.1. Study of colonial morphology

Isolated colonies of purified bacterial strains grown on solidified agar plates 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 (Pelczar and Reid, 1958).

2.3.2. Cellular morphology

Cells were observed with Gram staining (Duguid, 1989) 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.

2.3.3. Lactose fermentation

Lactose fermenting character in all the isolates was determined by streaking fresh broth culture on MacConkey agar (Oxoid) plates (Ng et al., 2010). Results were noted after incubation of 24 h at 37 °C.

2.3.4. Identification through GSP agar

The cultures were streaked on Glutamate Starch Phenol Red (GSP) agar (Oxoid) plates and incubated at 37 °C for 24–48 h (Martínez et al., 1998).

2.3.5. Heavy metal tolerance

All the isolates checked for heavy metal tolerance. To determine maximum tolerable concentration (MTC), 1 M stock solution of heavy metal salts copper, cadmium, lead, zinc, and nickel was prepared. Selective plates of Tris minimal media (Mergeay et al., 1985) supplemented with variable concentrations of heavy metal salts (Oxoid; 0.2 mM, 0.5 mM, 0.7 mM, 1 mM, 1.25 mM, 2 mM, and 3 mM) were utilized. MTCs were determined by streaking O/N culture on selective plates and results were observed after 24–48 h of incubation at 37 °C.

Figure 1. Different regions of sampling site including oil terminal, harbor, and undisturbed deep sea.
2.4. Screening methods for biosurfactant production

2.4.1. Hemolysis test
The first screening test for identification and isolation of biosurfactant-producing bacteria was the hemolysis test (Carrillo et al., 1996). Each isolate was streaked on blood agar medium and incubated at 37 °C for 24–48 h to assay for hemolytic activity. The plates were visually inspected for zones of clearing around the colonies, indicative of biosurfactant production.

2.4.2. Oil-spreading test
To apply the oil-spreading test, oil was layered over water in a petri plate and a drop of cell-free extract was added to the surface of oil (Morikawa et al., 2000). The diameter of the clear zone on the oil surface was measured in 3 replications for each isolate. A water drop was used as a negative control (Morikawa et al., 1993).

2.4.3. Emulsification index test
Emulsifying capacity of isolates was evaluated by an emulsification index (E24) for hexane, xylene, and crude oil. To do so, 1.5 mL of hydrocarbon was added to 1.5 mL of cell-free broth in a test tube, which was vortexed at high speed for 2 min and allowed to stand for 24 h. The percentage of the emulsification index was calculated using the following equation (Asfora Sarubbo et al., 2006):
\[ E24 = \text{Height of emulsion formed} \times 100/\text{total height of solution}. \]

2.4.4. Bacterial adhesion to hydrocarbons (BATH) assay
BATH assays were performed to estimate hydrophobicity of the cell surface as previously described by Rosenberg et al. (1980). The cell suspension (2 mL) with 100 µL of hydrocarbon (hexane, xylene, and crude oil) was added and vortexed and shaken for 3 min in test tubes (10 × 100 mm). After shaking, the hydrocarbon and aqueous phase were allowed to separate for 1 h. Reduction in turbidity of the aqueous phase was taken as the percentage of cell adherence to hydrocarbon, calculated as described by Van der Vegt et al. (1991):
\[ 1 - (\text{OD of the aqueous phase/OD of initial cell suspension}) \times 100. \]

2.4.5. Hydrocarbon overlay agar method
The hydrocarbon overlay agar method (Satpute et al., 2008) was performed with some modifications. Crude oil-coated LB agar plates were inoculated with O/N-grown culture of isolates and incubated at 30 °C for 48–72 h. A colony surrounded by emulsified halos was considered positive for biosurfactant production.

2.4.6. CTAB agar plate method
Blue agar plates containing cetyltrimethylammonium bromide (CTAB) (0.2 mg/mL) and methylene blue (5 mg/mL) were used to detect extracellular glycolipid production (Siegmund and Wagner, 1991). Biosurfactants were observed by the formation of dark blue halos around the colonies.

2.4.7. Drop-collapse test
Screening of biosurfactant production through drop collapse assay relies on the destabilization of a liquid drop on the hydrocarbon surface by cell-free extract containing biosurfactant. The assay was performed as described by Jain et al. (1991) and modified by Bodour and Maier (1998). Crude oil was spread on the lid of a petri plate and a drop of cell-free extract was placed on the hydrocarbon surface. A water was used as a negative control.

2.4.8. Emulsification assay
Cell-free culture broth was used as the biosurfactant source to check the emulsification of crude oil. First, 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 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 (DU 730 spectrophotometer, Beckman Coulter) 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 (Rosenberg et al., 1979). A negative control was maintained with only buffer solution and crude oil, and Triton X-100 was used as the positive control.

3. Results

3.1. Isolation of bacterial isolates
Totally, 89 bacterial colonies were isolated, purified, and screened for biosurfactant production. The strains were coded as DGHE1301–89.

3.2. Identification and characterization of isolates

3.2.1. Colonial morphology
The colonial morphology of all the isolates was observed: 14.6% were pinpointed, while the rest of the isolates had equal proportions of regular and irregular margins with variable sizes. Furthermore, 56.2% of the isolates had convex, 22.5% concave, and 21.3% flat elevation of the colony. Most of the colonies had a smooth and shiny texture while only 6.7% had rough texture. Finally, 84.2% were opaque and only 13.5% produced color pigment.

3.2.2. Cellular morphology
Cellular morphology, such as arrangement, shape, and Gram reaction, was observed: 55% were cocci and the rest of the isolates were rods. Furthermore, 38% had scattered cellular arrangement, 50% were in bunches, and 12% were arranged in chains. Most interesting were the Gram reactions, which revealed that isolates were 70% gram-negative and 30% gram-positive.
3.2.3. Lactose fermentation
Growth on MacConkey agar indicated that 48% of isolates had lactose-fermenting capability while 22% were nonlactose-fermenting colonies that appeared transparent and colorless. Meanwhile, 30% had no growth on MacConkey agar plates.

3.2.4. Identification through GSP agar
Selective plates of GSP agar revealed that among the 89 isolates, 28.1% were *Pseudomonas aeruginosa* and 6.74% were *Aeromonas hydrophila*.

3.2.5. Heavy metal tolerance
Results revealed none of the isolate as sensitive, since all the isolates tolerated 0.5 mM concentration of all the heavy metals tested. Cadmium was the most tolerable heavy metal salt; 92% of the isolates had MTCs of 3 mM against cadmium. Meanwhile, 67% of the isolates showed MTCs of 2 mM for zinc, and MTCs of 1.5 mM for copper, lead, and nickel were exhibited by 92%, 79%, and 82% of isolates, respectively. Results are shown in Figure 2.

3.3. Screening methods for biosurfactant production

3.3.1. Hemolysis test
The hemolytic activity was observed; results showed 24% positive for hemolysis. Out of all the isolates capable of it, 10% showed α-hemolytic activity and 14% showed β-hemolytic activity. Results are shown in Figure 3.

3.3.2. Oil-spreading test
From 89 strains, 44% of the isolates were positive for the oil-spreading assay and 15.7% showed the highest oil-spreading activity. Results are displayed in Figure 4.

3.3.3. Emulsification index test (E24)
Emulsification index of 30% or more was considered as significantly positive emulsification activity. Our study revealed that of 89 isolates, 68.5% showed positive emulsification activity with hexane, 46% showed positive emulsification activity with xylene, and 77.5% were miscible with crude oil. Meanwhile, 32.5% of the isolates showed positive emulsification to all the hydrocarbons and 6% did not show any emulsification to the 3 hydrocarbons tested. Results are exhibited in Figure 5.
3.3.4. BATH Assay
Hydrophobicity of the cell surface as estimated through BATH assay of 89 isolates revealed a maximum mean value of 23.4 ± 2.7% for hexane, followed by 20.1 ± 2.8% for xylene and 16.9 ± 3.1% for crude oil.

3.3.5. Hydrocarbon overlay agar method
The hydrocarbon overlay agar plate method revealed 65.1% of the isolates as positive for emulsified halos around the colony.

3.3.6. CTAB agar plate method
Of the 89 isolates, 52.8% produced dark blue halos around the colony and were considered as positive for CTAB agar plate assay. For zone formation, 1500 mm was the maximum size observed in isolate DGHE15.

3.3.7. Drop-collapse test
Drop-collapse test indicated positive results in 84% of the isolates.

3.3.8. Emulsification assay
Emulsification assay of isolates were performed with 3 hydrocarbons: hexane, xylene, and crude oil. Average emulsification units (EU/mL) results for hexane of 108 ± 8 EU/mL, for xylene of 200 ± 5 EU/mL, and for crude oil of 177 ± 10 EU/mL were recorded.

4. Discussion
The present study was aimed at the exploration of the indigenous microflora of the Karachi coast and the investigation of their biosurfactant-producing potential. Pollution of our seas, oceans, and coastal zones is a serious issue and contamination of hydrocarbons remains a major threat to the sustainability of the natural environment. Occurrence of biosurfactant-producing bacteria in hydrocarbon-polluted environments was reported by many researchers (Yateem et al., 2002; Bodour et al., 2003; Das and Mukherjee, 2005), considering which we selected the Karachi coastline of the Arabian Sea for the sampling site (Akhter et al., 2013).

Initial characterization of 89 isolated purified strains revealed multiple types of colonial and cellular morphologies. Predominant were gram-negative and lactose-fermenting bacterial isolates. About 28% of total isolates were identified as Pseudomonas aeruginosa through GSP agar plates. MTC of heavy metal salts was determined in order to select the strains with multiple metal resistances and those with high tolerance against the selected heavy metals. Stress resistance in isolates has clearly revealed the existence of contaminants at the sampling site (Akhter et al., 2013).

Hemolytic activity appears to be a good screening criterion in the search for biosurfactant-producing bacteria (Carter, 1984). In our studies, 24% of the isolates showed positive hemolytic activity, which is generally carried out as a primary method for screening of biosurfactant-producing bacteria. Other monitoring parameters that estimate surface activity, such as oil-spreadling test and the ability to emulsify hydrocarbons, are required for verification (Youssef et al., 2004). The oil-spreadling method is rapid and easy to carry out, requires no specialized equipment, and only requires a small volume of sample (Plaza et al., 2006). In this study, 44% of isolates were found to be positive by oil-spreadling method. Emulsification activity is one of the criteria to determine the potential of biosurfactants. Emulsifying activities (E24) determine the productivity of bioemulsifier (Bonilla et al., 2005) and are given as a percentage of the height of the emulsified layer divided by the total height of the liquid column. In the present study, only 6% of the total isolates showed negative emulsification potential, and almost 33% gave a good emulsification index with all 3 hydrocarbons tested, which included hexane, xylene, and crude oil. Ability of bacteria to adhere to hydrocarbons is a characteristic feature of biosurfactant-producing microbes. Rosenberg et al. (1980) developed the bacterial adhesion to hydrocarbons method, a simple photometrical assay for measuring the hydrophobicity of bacteria. The method is based on the degree of adherence of cells to various liquid hydrocarbons and we utilized 3 hydrocarbons for the assay. Isolates showed maximum adhesion to hexane. It is also possible to detect biosurfactant-producing and hydrocarbon-degrading activity simultaneously on agar plates by overlaying with hydrocarbon (Kokare et al., 2007). The hydrocarbon overlay agar method showed halos around the colony for 65.1% of isolates. The CTAB agar plate method is a semiquantitative assay for the detection of extracellular glycolipids or other anionic surfactants. The assay was developed by Siegmund and Wagner (1991) and in the present study revealed nearly 53% of isolates as positive. Jain et al. (1991) developed the drop-collapse assay. This assay relies on the destabilization of liquid droplets by surfactants. The drop-collapse method is a sensitive and easy to perform method and has several advantages in requiring a small volume of samples, being rapid and easy to carry out, and not requiring specialized equipment. The drop-collapse test was positive in 84% of the isolates. 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 (Belcher et al., 2012).
An interesting finding was that none of the isolates gave negative results in all screening tests, while only one gram-negative isolate, DGHE65, identified as *Pseudomonas aeruginosa*, was positive for all the tests for biosurfactant production. Successful isolation of a number of biosurfactant-producing bacteria may represent the ability of microorganisms to survive in hydrocarbon-contaminated regions and their ability to produce biosurfactants (Margesin and Schinner, 2001). These isolates represent naturally occurring stress-resistant bacteria successfully surviving in heavily contaminated regions and might be utilized as a productive tool for bioremediation in future (Shoeb et al., 2012). Microorganisms isolated in this study could be a valuable source of novel environmentally friendly biosurfactants for the future replacement of synthetic surfactants.

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**References**


