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Research Article

Isolation and Characterization of Biosurfactant Producing Bacteria Isolated from Produced Water

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Authors' Contributions
DS performed the experiments and wrote the manuscript. RAS supervised the sample collection. NJ designed, planned and supervised the study. RAS and NJ edited the manuscript.

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Biosurfactants, Produced water, Hydrocarbons, Emulsification.

Abstract | Biosurfactants are surface active naturally occurring compounds which are produced by microorganisms that have several applications in petroleum, pharmaceutical and agricultural industries. Produced water is a major waste water stream of petroleum industry which is produced during petroleum extraction from subsurface in which hydrocarbons are found as a main environmental pollutant. Present study is focused on production of biosurfactants from indigenous bacteria, isolated from produced water and produced water contaminated soil samples collected from three selected sites of Eastern Potwar, Punjab, Pakistan. Forty seven bacteria were isolated out of which five (F1, F3, F20, F23 and C16) were selected on the basis of high optical densities of 0.7, 70% oil and grease reduction potential and maximum CFU/ml of ≥3 $\times 10^6$. The weights in g/l of biosurfactants produced from F1, F3, F20, F23 and C16 were 0.52, 0.93, 1.58, 0.52 and 1.56 respectively. F20 showed maximum biosurfactant production of 1.58 g/l. F1, F3, F20, F23 and C16 showed emulsification (%) of 6.00, 6.33, 6.66, 6.00 and 6.33 and Rf-values of, 0.6, 0.61, 0.6, 0.64 and, 0.61 respectively. The GenBank accession numbers obtained for F1, F3, F20, F23 and C16 were MH424576, MH161599, MH424577, MH424578 and MH424579 respectively.

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Introduction

Surfactants are chemically synthesized compound that concentrates at the interface and lowers interfacial and surface tension. The majority of them are derived from petroleum (Banat *et al.,* 2000; Rosenberg *et al.,* 1999). Surface-active, naturally occurring molecules which-are produced by yeast, bacteria, and, fungi known as biosurfactants. Biosurfactants are, classified by molecular weight and chemical-composition. These chemical structures include fatty acids, glycolipids, phospholipid, peptides and glycopeptides (Cameotra *et al.,* 1998; Desai *et al.,* 1997; Kumar *et al.,* 2015). They are amphipathic compounds that are manufactured on the living-surfaces, mostly, on microbial-cell surfaces or are excreted as extracellular, hydrophilic and hydrophobic moiety. They have capacity of forming miscelles that collects at interface between fluids having different, polarities liquids. In this way they lessen surface and interface tension by obstructing the arrangement of hydrogen bridges and some hydrophobic and hydrophilic interactions. Thus possessing same property of lessening the interfacial and surface tension as that of chemical surfactants (Bicca *et al.,* 1999; Cunha *et al.,* 2004; Singh *et al.,* 2007).

Many microorganisms produce biosurfactants that includes Candida antartica, Acinetobacter species, Pseudomonas aeruginosa, and Bacillus species. Biosurfactants production is influenced by many factors like temperature, pH, aeration, nature of nitrogen and carbon source and C:N Ratio (Md, 2012). Attention towards biosurfactants has been generated because of its several applications re-

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lated to environmental protection, petroleum, crude-oil drilling, agriculture and pharmaceutical industries (Rahman *et al.,* 2008). Globally, there is concern about removal of hydrocarbons from environment produced by industrial activities and accidents like oil spills. Biosurfactants emulsifies the hydrocarbons (Bicca *et al.,* 1999). They possess the unique advantage of having low toxicity, high efficiency at intense temperature, salinity, pH, biodegradable nature and easy to synthesize. They are productively utilized as a part of dealing with industrial emissions, biodegradation of industrial effluents, oil slicks control and in bioremediation of polluted soil (Banat *et al.,* 2010; Mulligan, 2005; Patel *et al.,* 1997).

In this study, biosurfactant production potential of bacteria isolated from Produced Water (PW) and produced water contaminated soil samples has been evaluated. Produced Water is a major waste water stream of petroleum industry which is produced during petroleum extraction from subsurface having hydrocarbons as a main environmental pollutant. The main aim of this study was to select best biosurfactant producer bacterial strain among the isolated strains.

Materials and Methods

Sample Collection

Forty seven hydrocarbon utilizing indigenous bacteria were isolated, from produced water and produced water contaminated soil-samples which were collected from three sites of Eastern Potwar region (coordinates: 33° 30' 00" N, 73° 00' 00" E in degree minutes seconds), Punjab, Pakistan. The bacteria were isolated on nutrient agar medium (Priya *et al.,* 2009; Todar, 2009). Four produced water samples were collected from all three sites naming Site 1, Site 2A, Site 2B and Site 3. Two samples were collected from Site 2 and one (each) sample was collected from Site 1 and 3 respectively. Two soil samples were collected from each site.

Screening of Bacterial Strains

Oil and grease content (Ahmed Khadam *et al.,* 2009), and viable bacterial count (Colony forming unit CFU/ml) in produced water from all sites were calculated. A laboratory scale experiment was conducted to observe the growth (in terms of optical densities) of all isolated bacterial strains and their oil and grease reduction potential. Site 1 produced water gave highest oil and grease content of 1660mg/l as compared to the oil and grease content of Site 2A, 2B and Site 3 produced water which was 1460mg/l, 740mg/l and 880 mg/l respectively. Bacterial growth of all strains in terms of optical densities was observed by inoculating them in produced water of Site 1. The strains were incubated separately in different test tubes at 37°C and 120 rpm under shaking conditions for 144 hours. Maximum optical densities of 0.7,70% oil and grease reduction potential and maximum CFU/ml of ≥3 $\times 10^6$ was observed in five screened strains named as F1, F3, F20, F23 and C16.

Screening of Biosurfactant Activity

Haemolysis Test

The selected five strains were plated on blood agar plate to check the haemolytic activity at 37°C for 72 hours (Bicca *et al.,* 1999; Mulligan *et al.,* 1984; Tabatabaee *et al.,* 2005).

Growth of Screened Strains in Mineral Salt Media

The selected strains were inoculated for 48 hours in mineral-salt media. Mineral-salts media used in present study for screening of biosurfactant activity was a modification of mineral salts media used by Tabatabaee *et al.,* 2005. The media has the following composition: K_2HPO_4; 5g, KH_2PO_4; 20g, NaCl; 0.1g, $MnSO_4.7H_2O$; 0.22g, $(NH_4)_2SO_4$; 30g, $FeSO_4.7H_2O$;0.01 g, $CaCl_2.2H_2O$;0.02g, $MgSO_4.7H_2O$; 0.2g, Glucose; 1% and distilled water up to 1000ml. The pH of media was maintained at 7.2 (Tabatabaee *et al.,* 2005).

Emulsification Index Test (E24)

A mixture of cell free supernantants of all selected strains (2ml each) were taken in separate test tubes with 2ml of hydrocarbon (oil). The mixtures were vortexed for two minutes and left at room temperature for 24 hours. The percentage of E24 index was calculated by the given equation:

E24= Height of Emulsion formed/Total height of Solution × 100 (Barakat *et al.,* 2017; Techaoei, 2007)

Oil Spreading Technique

The 50ml distilled water was added to a petri plate having 10.16 cm (diameter). 20 µl of vegetable oil was added to the surface of distilled water with the addition of 10 µl of supernatant of culture broth (Rodrigues *et al.,* 2006; Priya *et al.,* 2009).

Drop Collapse Assay

The 5µl supernatant of culture broth was added to 2 µl of vegetable oil on a flat surface. Drop shape on oil surface was analysed after 1 minute. Flat (collapsed) drop showed the presence of biosurfactants while round drop means biosurfactant is absent (Barakat *et al.,* 2017; Youssef *et al.,* 2004).

Tilted Glass Slide Test

Selected bacterial strains were grown on nutrient agar plates for 24 hours. Single colony of each strain was taken and mixed with 0.85% of NaCl (droplet) at one end of a glass slide. Droplet of NaCl was observed by tilting the glass slide. The collapsing down of droplet showed the

presence of biosurfactants (Nwaguma *et al.,* 2016; Satpute *et al.,* 2010).

Extraction of Biosurfactants

Selected strains were inoculated for 48 hours in mineral-salt media. Biosurfactant were extracted from cell-free broths. Bacterial-cells were removed from broth, by centrifugation at 4ºC at 6000rpm for 20 min. pH of cell-free broth was adjusted to 2.0 with concentrated HCl. Broths were kept overnight at 4ºC and were again centrifuged for 20 min at 6000rpm. Cell free broths were discarded. Biosurfactants were extracted by adding chloroform:methanol (2:1) in the pallets, dried and their weights were calculated (Abu-Ruwaida *et al.,* 1991; Ghribi *et al.,* 2011; Priya *et al.,* 2009).

Analytical Approach: Thin Layer Chromatography (TLC)

Biosurfactants were analyzed by TLC. Biosurfactants were separated on TLC plate by using chloroform as solvent system with different reagents as iodine vapours and 20% H_2SO_4spray for color development (Abu-Ruwaida *et al.,* 1991; Priya *et al.,* 2009; Tabatabaee *et al.,* 2005).

Molecular Approach: 16s rRNA Sequencing

Selected bacterial-strains were identified by 16S rRNA gene sequencing from Macrogen. Sequence similarities search were made for the 16SrRNA sequences of F1,F3, F20, F23 and C16 using BLAST.

Results and Discussion

Isolation of Selected Strains

The selected strains showed maximum optical density of 0.7 (at 600 nm) with 70% oil and grease reduction potential in PW of Site 1. Cellular morphologies (Figure 1) of screened strains were studied through Gram staining technique. F1 and F3 were Gram negative bacilli, F20, F23 and C16 were Gram positive bacilli.

The present study evaluated the presence of biosurfactant producing bacteria in hydrocarbon contaminated sites. Many researchers reported the distribution and isolation of bacteria (biosurfactant producers)from hydrocarbon-contaminated and noncontaminated sites (Bodour *et al.,* 2003; Zou *et al.,* 2014). Bodour and Miller-Maier showed that hydrocarbon contaminated sites are more yielding in biosurfactant producing bacteria than noncontaminated sites (Bodour *et al.,* 1998).

Molecular Approach

The 16S rRNA studies of organisms showed 99% similarities with *Acinetobacter baumannii*, *Acinetobacter baumannii*, *Bacillus thuringiensis*, *Bacillus cereus* and *Bacillus subtilis* for F1, F3, F20, F23 and C16 respectively in Nucleotide data-base of National Centre for Biotechnological Information (NCBI). The GenBank accession numbers obtained for F1, F3, F20, F23 and C16 are MH424576, MH161599, MH424577, MH424578 and MH424579 respectively.

Screening of Biosurfactant Activity

Haemolysis Test

Hemolytic activity was observed in all the five selected strains. Haemolysis is regarded as indicative test for biosurfactant production and is used as rapid method for screening of bacterial strains (Banat, 1995; Carrillo *et al.,* 1996; Deepak *et al.,* 2015; Mulligan *et al.,* 1984; Tabatabaee *et al.,* 2005).

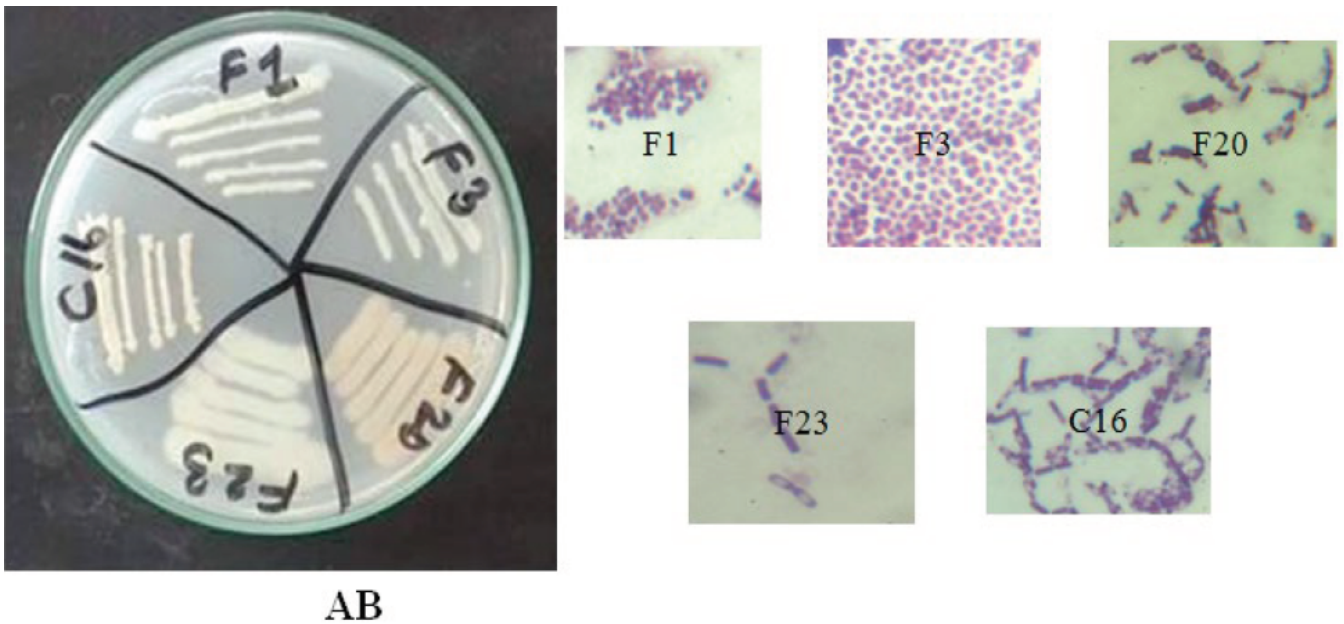

Figure 1: A represents five selected Bacterial Strains. B represents Cellular Morphology of Bacterial Strains under Light Microscopy. The Gram stained bacterial strain of variable sizes are visible. B1: *Acinetobacter baumannii* **(F1),**

B2: *Acinetobacter baumannii* **(F3), B3:** *Bacillus thuringiensis* **(F20), B4:***Bacillus cereus* **(F23), B5:** *Bacillus subtilis* **(C16).**

Table 1: Screening Tests for Biosurfactants

Strains	Haemolysis Activity	Emulsification Index (EI) %	Oil Spreading Assay (cm)	Drop Collapse Assay	Tilted Glass slide Test	Weights of Biosurfactants (g/l)	Rf values of Biosurfactants
F1	+	6.00	0.5	+	+	0.52	0.6
F3	+	6.33	0.49	+	+	0.93	0.61
F20	+	6.66	0.6	+	+	1.58	0.6
F23	+	6.00	0.56	+	+	0.52	0.64
C16	+	6.33	0.59	+	+	1.56	0.61

Emulsification Index test (E24)

High emulsification of 6.66% was observed in F20 bacterial strain. Table 1 shows the emulsification index of all five strains. Many studies focused on high emulsifying abilities. The stabilization of oil and water emulsion is mainly used as surface activity indicator after haemolysis test (Bodour et al., 2004; Francy et al., 1991). Emulsification index and oil spreading techniques are quantitative techniques. The quantity of biosurfactant production is correlated with their values (Bicca et al., 1999; Nwaguma et al., 2016).

Oil spreading technique

All five strains showed positive result with oil spreading technique. F20 (*Bacillus thuringiensis*) showed maximum zone with diameter of 0.6cm. Zone formation by all five strains is shown in Table 1. Priya *et al.* studied biosurfactant activity with vegetable oil in *Bacillus subtilis*. *Bacillus subtilis* produced the high zone formation with diameter 0.6cm with vegetable oil. In our study Bacillus Subtillis displayed a zone of 0.59 cm with vegetable oil which is almost similar to reported values (Priya et al., 2009).

Drop collapse assay

The selected five strains showed positive results in this test. Drop collapse assay is a qualitative technique for detection of biosurfactants (Deepak *et al.,* 2015). According to Satpute *et al.,* for identification of all types of biosurfactants, a single method is not suitable so a combination of different methods are recommended (Satpute *et al.,* 2008).

Tilted glass slide test

The screened strains showed positive results in tilted glass slide test (Table 1). This test is qualitative test for identification of biosurfactants producers. Previously the following methods have-been used for identification of biosurfactant-producing bacteria; tilted glass slide (Bodour *et al.,* 1998; Satpute *et al.,* 2008), emulsification index (Mathai *et al.,* 2001), haemolytic activity (Carrillo *et al.,* 1996; Satpute *et al.,* 2010) and oil-spreading technique (Satpute *et al.,* 2008).

Extraction of biosurfactants

The biosurfactants, were extracted by using acid-precipitation-method at pH 2. The mean values of weights of biosurfactants produced from F1, F3, F20, F23 and C16 were 0.52, 0.93, 1.58, 0.52 and 1.56 g/l respectively. Maximum biosurfactant-production of 1.58 g/l was obtained from F20.

Thin layer chromatography

The Rf values of biosurfactants produced, by F1, F3, F20, F23 and C16 were 0.6, 0.61, 0.6, 0.64 and 0.61 respectively (Figure 2). Sodium dodecyl sulphate (SDS) was used as standard.

According to Tabatabaee *et al.* (2005) the 0.6 Rf value indicates the presence of glycolipid or neutral lipids. In present study chloroform was used a solvent system. Tabatabaee et. al., 2005 used chloroform-methanol-aceticacid-water mixture (25:15:4:2), Abu Ruwaida et. al., 1991 used chloroform-methanol-acetic acid mixture (80: 15: 5) as solvent systems.

Figure shows TLC for biosurfactants. 1,2,3,4 and 5 represents spots of biosurfactants produced by bacterial strains F1, F3, F20, F23 and C16 with Rf values 0.6, 0.61, 0.6, 0.64 and 0.61 respectively observed under ultraviolet light at 365 nm. Spot 6 represents sodium dodecyl sulphate (SDS) used as standard. The upper most layer represents solvent front.

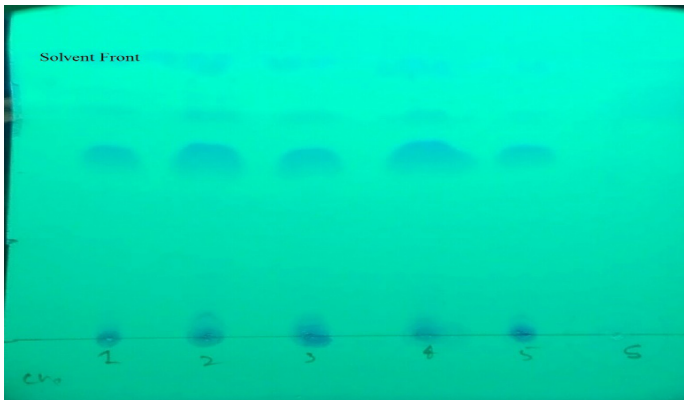

Figure 2: Analytical Analysis: Thin Layer Chromatography (TLC) for Biosurfactants

Conclusions and Recommendations

Present study shows efficient production of biosur-

factants from indigenous bacteria, isolated from hydrocarbon contaminated produced water and soil collected from Eastern Potwar, Punjab, Pakistan. The ability of isolated bacteria from this Potwar region is very important for the production of biosurfactants considering the level of hydrocarbon pollution and the need to use ecologically friendly and indigenous products for remediation processes. Out of 47 bacterial strains five were selected. All were biosurfactant producers among which F20 showed maximum biosurfactant production of 1.58 g/l and maximum emulsification index of 6.66%. Further research work is in progress.

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Statement of Conflict of Interest

The authors declare no conflict of interest.

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