Biosurfactant production by bacteria retrieved from hydrocarbon polluted environment

Kavita Rani, Seema Sangwan, Sushila Singh, Pankaj Sharma, Harpreet Kaur and Meena Sindhu

DOI: https://doi.org/10.22271/chemi.2020.v8.i4h.9744

Abstract

Bacterial isolates retrieved from stagnant water and hydrocarbon polluted soil using enrichment culture technique were studied for biosurfactant production. Sixteen isolates, showing blood agar haemolysis, were screened for biosurfactant production. Highest oil displacement was generated by isolate BK68 (0.0623 m) followed by 0.0248 m, 0.0223 m and 0.0206 m by isolates BK23, BK34 and BK48, respectively. Highest ESI index was given by isolate BK66 (19.5%) followed by BK68, BK58 and BK23 (19.0%, each) after 4 days of incubation. Surface tension reduction equivalent to 0.071 Nm⁻¹, was shown by isolate BK68, followed by BK23 (0.037 Nm⁻¹) and BK66 (0.036 Nm⁻¹). Promising biosurfactant producers were subjected to identification to genus level. Gram positive and endospore forming isolates BK23, BK58 and BK68 were probably Bacillus spp, while catalase positive cocci bacteria BK66 could be classified as Staphylococcus. Gram negative, oxidase positive and non-glucose fermenting rods of isolates BK34 and BK48 were probably belong to genus Pseudomonas.

Keywords: Enrichment culture technique, Haemolysis, Emulsification index, Surface tension reduction, Oil displacement, Oil atomization

Introduction

Soil and water environments are frequently contaminated with oil hydrocarbons (OHC), polyaromatic hydrocarbons (PAH) and other hydrophobic substrates which often lead to severe environmental consequences. Microbial consortia display a wide array of metabolic mechanisms including production of emulsifiers and biosurfactants as one fairly effective strategy, for bioremediation of such pollutants (Parthipan et al 2017) [15]. Biosurfactants are surface active metabolites containing hydrophobic and hydrophilic moieties that reduce surface and liquid-liquid or solid-solvent interfacial tensions (Shahaby et al. 2015) [20] and enhance solubilization of hydrocarbons into water which eventually leads to better degradation of these pollutants. Besides, these surface active compounds are also having applications in enhanced oil recovery, food processing, pharmaceuticals etc. that can be exploited commercially (Santos et al. 2016) [18].

Many biosurfactant producing microorganisms, particularly bacteria and yeasts, are reported in literature such as Pseudomonas spp., Bacillus spp., Lactococcus lactis, Lactobacillus strains, Streptococcus thermophilus, Nocardioides spp., Aeromonas spp., Serratia spp., Rhodococcus strains and Candida ingens (Chen et al. 2007; Khopade et al. 2011; Rodrigues et al 2006; Sharma et al. 2019) [9, 13, 10, 21]. These are mainly abundant in soil or water samples contaminated with hydrophobic organic compounds like refinery wastes (Batista et al. 2006) [3], hydrocarbon polluted areas (Bento et al. 2004) [4] and marine environments (Antoniou et al. 2015) [1].

Currently, very few biosurfactants are commercially available e.g., surfactin, sophorolipids and rhamnolipids and the enormous market demands for surfactants are generally met by numerous synthetic mainly petroleum based, chemical surfactants which are usually non-degradable and toxic to the environment (Banat et al. 2000). Moreover, biosurfactants are more effective and versatile than many synthetic surfactants owing to their selective action, biodegradable nature and stability at high temperature, pH and salinity. The development of this line of research is of paramount importance, mainly in view of the present concern with protection of the environment.
Keeping in view, the commercial application of biosurfactants continuous efforts are required for unearthing superior biosurfactant producing microbial strains. The present investigation, therefore, was undertaken with the objectives of isolation, screening and identification of biosurfactant producing bacteria form hydrocarbon contaminated environment.

Materials and Methods
Collection of Water and Soil Samples
Water sample was collected in form of water surface microlayer (Wsmu) by the glass plate method from multiple locations of a pond having stagnant water. A plexiglass plate was disinfected with 70% ethanol and immersed slowly in an upright position for 1 minute at water sampling site. The plate was then removed gently in the same position and allowed to drip for 30 seconds. The water adhering to both surfaces was wiped-off into a sterilized glass petri plate (0.15 m) by forcing the plate between two teflon wiper blades before collecting in a sterilized reagent bottle. In addition to this, total 5 soil samples were also collected in sterile plastic zip bags from hydrocarbon contaminated sites of petrol pumps and automarket. All the samples were stored at room temperature until intended use.

Isolation of Biosurfactant Producing Bacteria
The water and soil samples were inoculated at the rate of 1% (v/v)/(w/v) for enrichment in mineral salt (MS) broths prepared by mixing, one liter of solution ‘A’ containing per liter, 2.5 g NaNO₃, 0.4 g MgSO₄·7H₂O, 1.0 g NaCl, 1.0 g KCl, 0.05 g CaCl₂·2H₂O and 10.0 ml of 85% H₃PO₄ with 1.0 ml of solution ‘B’ containing per liter, 0.5 g FeSO₄·7H₂O, 1.5 g ZnSO₄·7H₂O, 1.5 g MnSO₄·2H₂O, 0.15 g CuSO₄·5H₂O and 0.1 g NaMnO₄·2H₂O (pH-7.2, adjusted with KOH pellets). The MS broth was also supplemented with 1% (v/v) of either liquid paraffin, crude oil or commercial diesel in separate treatments before incubating at 120-130 rpm for one week. Sub-enrichment was repeated minimum three times by transferring 10 ml of enriched sample into fresh 90 ml MS broth of similar composition. Alternatively, samples without any enrichment were also used for isolation of bacteria. Isolation was done by streaking enriched broth on nutrient agar (NA) plates. Isolates were picked up after 24-48 h of incubation either depending upon difference in colony morphology or on the basis of appearance on different NA plates inoculated with variably enriched samples, purified by repeated streaking for 3-4 times and maintained on NA slants at 4°C for further use.

Screening of Bacterial Isolates for Biosurfactant Production
All the isolates were first screened on the basis of blood agar haemolysis (Plaza et al. 2006) [16]. Bacterial isolates giving positive haemolytic test were screened on the basis of oil displacement technique (Kaur et al. 2017) [12] with a modification. The method was modified in this study, by using mobile oil to overlay the water instead of almost colourless diesel or petrol so as to clearly visualize the oil displacement in a blackish red background. Other screening methods used for selection of biosurfactant producers were emulsification (E₂₄) index (Sarubbo 2006) [19], surface tension reduction (Suganya 2013) [22] and atomized oil assay (Burch et al. 2011) [3]. For all these methods, 24 h grown bacterial slant was transferred to 100 ml sterilized nutrient broth and incubated at 120-130 rpm. Ten milliliter of 15-16 h grown culture (of almost equal Aₖ₅₄₅ nm) was transferred to 50 ml MS broth supplemented with 2% (w/v) glucose as carbon source and incubated at 160 rpm for 6 days. One flask of each culture was withdrawn after every 24 h and centrifuged at 6000 rpm for 15 minutes. The cell free supernatants were used in various screening methods for biosurfactant production and the results were compared with 0.2% SDS as positive control and distilled water as negative control. All the experiments were performed at 30±2°C unless it is specified.

Identification of Promising Isolates
Promising isolates were identified up to genus level on the basis of morphological, cultural and biochemical characteristics (Bergey 1989) [6]. Cultural characteristics were studied by inoculating bacterial isolates on NA plates to record form, margin and elevation of colonies and single line streak on NA slant to observe their growth, pigmentation, opacity and form, for 24 h. Alternatively, nutrient broth was inoculated with selected isolates for observation of type and amount of growth under stationary condition. Morphological and biochemical characteristics were studied by negative, spore and Gram staining techniques as per standard methods. Catalase test was performed by adding 20 µL of 3% H₂O₂ to 24 h grown, broth of bacterial isolates taken on a clean slide where emission of effervescences was recorded as positive catalase test. Oxidase test was performed by adding few drops of 1% freshly prepared Wruster’s reagent containing 1.0 g of N,N,N’,N’-tetramethyl-p-phenylene diamine dihydrochloride in distilled water into 24 h old bacterial broth. Alternatively, 24 h grown bacterial isolates were streaked over the oxidase discs (Hi-media). Immediate appearance of deep blue to purple Colour was recorded as positive oxidase enzyme activity. Glucose fermentation was studied by inoculating 24 h old bacterial isolates into glucose fermentation broth of pH 6.8-7.0 containing per liter, 5.0 g peptone, 3.0 g beef extract, 10.0 g glucose and 1.0 ml of phenol red as indicator at 120 rpm for 24-48 h. Change in Colour of the broth to pale yellow was recorded as a positive test.

Results
Isolation of Biosurfactant Producing Bacteria
Spontaneous release and function of biosurfactants are often related to hydrocarbon uptake; therefore, hydrocarbon contaminated sites and other polluted areas are considered as most promising locations for isolation of these organisms (Bento et al. 2005) [14]. Total 78 bacterial isolates, including 16 from water surface microlayer (Wsmu) of a polluted stagnant pond and 62 from hydrocarbon contaminated soil samples were retrieved. Total 63 isolates were selected from enriched treatments having crude oil, liquid paraffin and diesel as carbon sources while 15 isolates were from non-enriched soil samples in ‘as-is’ condition (Table 1).
Table 1: List of samples and their sampling sites

| Samples and Location details | Enrichment details | No. of bacterial isolates | Total no. of isolates |
|-----------------------------|-------------------|--------------------------|-----------------------|
| Water Surface Microlayer (W SmL): Pond, CCS HAU, Hisar (Latitude 29° 08’.41.2”N and longitude 72° 42.25.8”E) | W SmL + Crude oil | 8 (BK1, BK2, BK3, BK4, BK5, BK6, BK7 and BK48) | 16 |
| Soil 1 + Diesel | 2 (BK13 and BK14) |
| | W SmL + Liquid paraffin | 6 (BK8, BK9, BK10, BK11, BK12 and BK49) |
| Soil 1 + Diesel | 12 (BK24, BK25, BK26, BK27, BK28, BK29, BK30, BK31, BK32, BK33, BK34 and BK51) |
| Soil 2: Petrol pump, CCS HAU, (Latitude 29° 9’,10.01”N and longitude 75° 41’.51.64”E) | Soil 1 + Diesel | 5 (BK15, BK16, BK17, BK18 and BK19) |
| Soil 2 + Liquid paraffin | 5 (BK20, BK21, BK22, BK23 and BK50) |
| Soil 2 + Crude oil | 1 (BK41) |
| Soil 3: Automarket, Hisar: Location 1 (Latitude 29° 10’,1,13”N and longitude 75° 43’.37.32”E) | Soil 3 + Liquid paraffin | 5 (BK42, BK43, BK53, BK54 and BK55) | 8 |
| Soil 3 + Crude oil | 1 (BK56) |
| Soil 4: Automarket, Hisar: Location 2 (Latitude 29° 10’,7.92”N and longitude 75° 43’.30.13”E) | Soil 4 + Liquid paraffin | 1 (BK69) | 11 |
| Soil 4 + Diesel | 3 (BK60 and BK61) |
| Soil 4 + As is’ | 4 (BK62, BK63, BK64 and BK65) |
| Soil 5: Automarket, Hisar: Location 3 (Latitude 29° 9’,55.98”N and longitude 75° 43’.39.25”E) | Soil 5 + Diesel | 3 (BK70, BK71 and BK72) | 10 |
| Soil 5 + Liquid paraffin | 1 (BK76) |
| Soil 5 + As is’ | 6 (BK44, BK45, BK46, BK47, BK73 and BK74) |
| Soil 6 | 2 (BK77 and BK78) |
| Total No. of bacterial isolates | 78 |

Screening of Bacterial Isolates for Biosurfactant Production

Several protocols have been designed and listed in literature to demarcate biosurfactant producers in a general microbial population. Blood agar hemolysis is considered as simpler and preliminary method to testify biosurfactant production ability (Thavasi et al. 2011a) [24]. Total 16 isolates have shown blood agar hemolysis after 24-48 h of incubation (Fig. 1A). Five out of total 16 isolates were obtained from water surface microlayer while rest 11 bacterial isolates were retrieved originally from enriched soil samples. Since haemolytic activity could also be shown by several other metabolites; therefore, this method has to be supported by other available screening methods. Therefore, these 16 isolates were subjected to further screening using oil displacement technique, E24 index, surface tension reduction and atomized oil assay for stronger confirmation of biosurfactant production. Use of mobile oil instead of diesel during oil displacement technique clearly visualized oil displacement, in a coloured background (Fig. 1B). Bacterial isolates BK68, BK23, BK34 and BK48 were found to be giving significant displacement of oil equivalent to 0.0543, 0.0248, 0.0223 and 0.0206 m after 4 days of incubation. Isolate BK68 gave highest oil displacement, 0.0623 m after 5 days of incubation which was found comparable to positive control, 0.2% SDS (0.0660 m) as detailed in table 2.

| Bacterial isolates | Oil displacement (cm) | Emulsification (E24) index (%) |
|-------------------|-----------------------|--------------------------------|
|                   | Incubation period (days) | 1  | 2  | 3  | 4  | 5  | 6  | 1  | 2  | 3  | 4  | 5  | 6  |
| BK3               | 0.50 0.83 1.03 1.13 1.16 1.16 | 5.0 | 5.0 | 7.5 | 10.0 | 12.5 | 12.5 |
| BK5               | 0.91 1.03 1.15 1.25 1.25 1.24 | 10.0 | 12.5 | 14.0 | 17.0 | 17.5 | 17.5 |
| BK13              | 0.73 1.20 1.18 1.26 1.26 1.25 | 12.5 | 12.5 | 12.5 | 12.5 | 15.0 | 14.0 |
| BK16              | 0.46 0.81 0.91 1.23 1.22 1.22 | 5.0 | 5.0 | 7.5 | 9.0 | 9.0 | 9.0 |
| BK23              | 0.81 1.41 1.91 2.48 2.45 2.44 | 7.5 | 13.0 | 17.5 | 19.0 | 19.0 | 18.0 |
| BK34              | 1.36 1.25 1.51 2.23 2.21 2.20 | 9.0 | 12.5 | 17.5 | 18.0 | 18.0 | 18.0 |
| BK35              | 1.21 1.23 1.31 1.51 1.50 1.49 | 7.5 | 10.0 | 12.5 | 14.0 | 14.0 | 14.0 |
| BK38              | 1.35 1.43 1.48 1.51 2.36 2.36 | 10.0 | 10.0 | 10.0 | 14.0 | 14.0 | 14.0 |
| BK41              | 0.73 1.00 1.18 1.20 1.21 1.21 | 12.5 | 12.5 | 14.0 | 14.0 | 15.0 | 15.0 |
| BK48              | 1.90 1.90 2.00 2.06 2.06 2.06 | 10.0 | 12.5 | 15.0 | 15.0 | 15.0 | 14.0 |
| BK49              | 0.86 1.00 1.23 1.28 1.28 1.27 | 5.0 | 7.5 | 10.0 | 12.5 | 12.5 | 12.5 |
| BK50              | 1.18 1.20 1.26 1.26 1.25 1.24 | 7.5 | 12.5 | 15.0 | 15.0 | 15.0 | 15.0 |
| BK51              | 1.00 1.16 1.20 1.63 1.53 1.53 | 7.5 | 10.0 | 15.0 | 17.5 | 17.5 | 16.0 |
| BK58              | 1.25 1.25 1.28 1.36 1.36 1.36 | 15.0 | 15.0 | 17.5 | 19.0 | 19.0 | 19.0 |
| BK66              | 0.70 0.93 1.06 1.18 1.18 1.17 | 15.0 | 15.0 | 17.0 | 19.5 | 19.5 | 19.5 |
| BK68              | 1.43 2.53 3.83 5.43 6.23 6.23 | 10.0 | 14.0 | 17.5 | 19.0 | 20.0 | 20.0 |
| 0.2% SDS          |                          | 6.60 |                | 37.5 |
| Distilled water   |                          | 0.0  |                | 0.0  |

“1061”
Isolate BK68 gave highest 20.0% $E_{24}$ index on 5th day of incubation (Table 2 and Fig. 1B) followed by isolate BK66 (19.5%) and BK58, BK23 (19.0%, each) after 4 days of incubation. Oil atomization was studied using a fine spray of mineral oil which created light-diffractive halos around the equilibrated culture supernatant on LB agar plates. The radii of halos were measured from outer surface of culture supernatant up to halos formation. On 4th day of incubation isolate BK68 gave highest oil atomization up to 0.0043 m followed by isolates BK23 (0.0040 m), BK34 (0.0038 m) and BK66 (0.0035 m), however, the radii of halo further increased to 0.0050 m on 5th day of incubation in case of isolate BK68.

Identification of Promising Isolates

All the six selected isolates were subjected to identification on the basis of determination of morphological, cultural and biochemical analysis. Colony appearances were found to be circular.
except BK23 and BK68 which were having irregular colonies on NA plates. Margins appeared undulate in isolate BK68, serate in isolates BK23 and BK68 and entire in isolates BK34, BK48 and BK66. All the isolates were having flat elevations except BK66 which was having raised elevation. Growth of isolate BK34 was found scanty while all the other isolates were having moderate to abundant growth on NA slants. All the isolates were non-pigmented except BK66 which was having pink pigmentation. Single line streak on NA slants was found to be filiform except in case of isolates BK34 and BK58 which were having beaded and echinulate forms of growth, respectively. Growth of isolates in nutrient broth was found to be pellicle in isolates BK23, BK34 and BK48, sedimented in isolate BK58 and uniform type in isolate BK68 at 30+2ºC.

Morphologically all the selected isolates were found to be uniform rods except BK66 which was found to be a coccus, as observed by negative staining. Gram staining indicated isolates BK23, BK58, BK66 and BK68 as Gram positive while isolates BK34 and BK48 were found to be Gram negative (Fig.3A-F). Further, bacterial isolates BK23, BK58 and BK68 were observed as endospore formers while BK66 was found to be a non-spore former (Fig.3G-I). Conclusively, aerobic, Gram positive endospore forming bacterial isolates BK23, BK58 and BK68 were unspecified species belonging most probably to genus Bacillus.

Catalase test was examined to determine the presence of catalase activity. Emission of effervescences after addition of few drops of 3% H₂O₂ indicated BK66 as catalase positive. Since BK66 was a Gram positive coccus (in bunches), catalase positive, showing β-haemolysis and pink pigmentation, therefore, the isolate is expected to be of genus Staphylococcus.

To identify Gram negative bacterial isolates, oxidase test was conducted using Wruster’s reagent. Isolate BK34 and BK48 gave deep blue colour within 5-10 seconds indicating positive reaction as compared with negative control (Bacillus sp.), which gave the deep blue colouration after 30 seconds. Alternatively, oxidase activity using discs further confirmed the results (Fig.3 J-K). Glucose fermentation broths were incubated at 30ºC for 48h. Moreover, both the isolates BK34 and BK48 gave a negative glucose fermentation test. In conclusion, Gram negative rods of bacterial isolates BK34 and BK48 giving negative oxidase and glucose fermentation test, may be identified as Pseudomonas spp. (Table 3).
| Bacterial isolates | Colony appearance | Margination | Elevation (24h) | Pigment formation | Opacity | Type and amount of growth | Shape | Gram Reaction | Spore formation | Catalase Test | Blood Agar Haemolysis | Oxidase Test | Glucose Fermentation | Probable Genus |
|-------------------|-------------------|-------------|----------------|------------------|---------|---------------------------|-------|---------------|----------------|--------------|---------------------|-------------|----------------------|---------------|
| BK23              | Irregular         | Serate      | Flat           | Moderate         | White   | Opaque                    | Filliform Pellicle and Moderate rods | +ve | Endospore former | NA            | B            | NA                  | NA          | Bacillus spp.        |               |
| BK34              | Circular          | Entire      | Flat           | Scanty           | White   | Translucent Beaded       | Filliform Pellicle and Moderate rods | -ve | NA             | A             | +ve          | -ve                 | Pseudomonas spp. |               |
| BK48              | Circular          | Entire      | Flat           | Moderate         | White   | Opaque                    | Filliform Pellicle and Moderate rods | -ve | NA             | A             | +ve          | -ve                 | Pseudomonas spp. |               |
| BK58              | Circular          | Serate      | Flat           | Abundant         | White   | Opaque                    | Echinulate Sedimented and Abundant rods | +ve | Endospore former | NA            | B            | NA                  | NA          | Bacillus spp.        |               |
| BK66              | Circular          | Raised      | Moderate       | Pink             | Transparent | Filliform Uniform and Abundant coccus in banches | +ve | NA            | +ve            | B            | NA                  | Staphylococcus spp. |               |
| BK68              | Irregular         | Undulate    | Flat           | Moderate         | White   | Opaque                    | Filliform Sedimented and Moderate rods | +ve | Endospore former | NA            | B            | NA                  | NA          | Bacillus spp.        |               |

**Discussion**

Results of E54 index falls in a range of 9.0-20.0% however the isolates giving E54 above 18% were selected as promising isolates for further studies. *Bacillus* spp. and *Pseudomonas* spp. were reported to be giving E54 index with crude oil equivalent to 9.0% and 17.0%, respectively (Myla et al. 2010) [14]. Surface tension reduction, a confirmatory test for biosurfactant production, was measured by drop weight method using culture supernatant of all the 16 bacterial isolates. Seven out of the total 16 isolates which were tested positive for blood haemolysis test gave more than 0.030 Nm⁻¹ reduction in surface tension of culture medium, while maximum reduction in surface tension was observed in case of isolate BK68 (0.071 Nm⁻¹). These values of surface tension reduction suggested significant biosurfactant production particularly in case of isolate BK68 which is further comparable to some of the reports available in literature giving maximum reduction in surface tension equivalent to 46.5mNm⁻¹ (equal to 0.046 Nm⁻¹) by *Acinetobacter junii* (Bento et al. 2004) [5] and in the range of 28±1.03mNm⁻¹ to 51±1.36mNm⁻¹ (equal to 0.028±0.00103 Nm⁻¹ to 0.051±0.00136 Nm⁻¹) by *Bacillus amyloliquefaciens* (Thavasi et al. 2011) [23].

**Conclusion**

The ability of bacterial isolates, specifically isolate BK68, to produce significant level of biosurfactant as indicated by multiple screening techniques, suggests their potential for exploitation at commercial level. However, fermentation production of these biodegradable and ecofriendly surfactants is limiting due to their increased cost of production at large scale. Therefore, to improve the overall economy of the process, optimization of physical and nutritional parameters along with utilization of cheaper raw material is strongly recommended.

**Conflict of interest statement**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**References**

1. Antoniou E, Fodelianakis S, Korkakaki E, Kalogerakis N. Biosurfactant production from marine hydrocarbon-degrading consortia and pure bacterial strains using crude oil as carbon source. Front Microbiol. 2015; 6:274. http://doi:10.3389/fmicb.2015.00274.

2. Banat IM, Makkar RS, Cameotra SS. Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol. 2000; 53:495-508.

3. Batista SB, Mounteer AH, Amorim FR, Totola MR. Isolation and characterization of biosurfactant/bioemulsifier-producing bacteria from petroleum contaminated sites. Biorech Technol. 2006; 97:868-875.

4. Bento FM, Camargo FAO, Okeke BC, Frankenberger WT. Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. Microbiol Res. 2005; 160:249-255.

Table 3: Biochemical parameters of biosurfactant producing bacterial isolates

- **Bacterial isolates**: BK23, BK34, BK48, BK58, BK66, BK68
- **Colony appearance**: Circular, Irregular, Circular, Circular, Circular, Circular
- **Margination**: Serate, Entire, Entire, Serate, Raised, Undulate
- **Elevation (24h)**: Flat, Flat, Flat, Flat, Flat, Flat
- **Pigment formation**: White, Translucent, White, Opaque, Pink, White
- **Opacity**: Opaque, Opaque, Opaque, Opaque, Opaque, Opaque
- **Type and amount of growth**: Filliform, Filliform, Filliform, Filliform, Filliform, Filliform
- **Shape**: Pellicle and Moderate, Pellicle and Scanty, Pellicle and Moderate, Sedimented and Abundant, Uniform and Abundant, Sedimented and Moderate
- **Gram Reaction**: +ve, +ve, +ve, +ve, +ve
- **Spore formation**: Endospore former, NA, Endospore former, Endospore former, Endospore former
- **Catalase Test**: NA, NA, A, NA, B
- **Blood Agar Haemolysis**: NA, NA, NA, NA, NA
- **Oxidase Test**: A, +ve, A, +ve, -ve
- **Glucose Fermentation**: NA, NA, NA, NA, NA
- **Probable Genus**: Bacillus spp., Pseudomonas spp., Bacillus spp., Bacillus spp., Staphylococcus spp., Bacillus spp.
5. Bento FM, Camargo FAO, Okeke BC, Frankenberger WT. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Biorese Technol. 2004; 96:1049-1055.

6. Bergey DH. Bergey's Manual of Systematic Bacteriology. Williams & Wilkins publishers. 1989; 4:1.

7. Burch AY, Browne PJ, Dunlap CA, Price NP, Lindow SE. Comparison of biosurfactant detection methods reveals hydrophobic surfactants and contact-regulated production. Environ Microbiol. 2011; 13:2681-2691.

8. Chandankere R, Yao J, Masakorala K, Jain AK, Kumar R. Enhanced production and characterization of biosurfactant produced by a newly isolated Bacillus amyloliquefaciens USTBb using response surface methodology. Int. J Curr Microbiol Appl Sci. 2014; 3(2):66-80.

9. Chen SY, Wei YH, Chang JS. Repeated pH-staffed-batch fermentation for rhamnolipid production with indigenous Pseudomonas aeruginosa S2. Appl Microbiol Biotechnol. 2007; 76:67-74.

10. Gudina EJ, Fernandes EC, Rodrigues AI, Teixeira JA, Rodrigues LR. Biosurfactant production by Bacillus subtilis using corn steep liquor as culture medium. Front Microbiol. 2015; 6:59. http://doi:10.3389/fmicb.2015.00059.

11. Hu X, Wang C, Wang P. Optimization and characterization of biosurfactant production from marine Vibrio sp. strain 3B-2. Front Microbiol. 2015; 6:976. http://doi:10.3389/fmicb.2015.00976.

12. Kaur K, Sangwan S, Kaur H. Biosurfactant production by yeasts isolated from hydrocarbon polluted environments. Environ Monit Assess. 2017; 189:603. https://doi.org/10.1007/s10661-017-6311-x.

13. Khopade A, Biao R, Liu X, Mahadik K, Zhang L, Kokare C. Production and stability studies of the biosurfactant isolated from marine Nocardicopsis spp. B4. Desalination. 2011; 285:198-204.

14. Myla J, Chandrasekaran R, Muthu RS. Screening optimization and production of biosurfactants from Bacillus and Pseudomonas spp. Biomed Pharmacol J. 2010, 3(1).

15. Parthipan P, Preetham E, Machuca LL, Rahman PK, Murugan K, Rajasekar A. Biosurfactant and degradative enzymes mediated crude oil degradation by bacterium Bacillus subtilis A1. Front Microbiol. 2017; 8:193. http://doi:10.3389/fmicb.2017.00193.

16. Plaza GA, Zjawiony I, Banat IM. Use of different methods for detection of thermophilic biosurfactant producing bacteria from hydrocarbon-contaminated and bioremediated soils. J Pet Sci Eng. 2006; 50:71-77.

17. Rodrigues LR, Teixeira JA, Mei HC, Oliveira R. Physiochemical and functional characterization of a Biosurfactant produced by Lactococcus lactis colloids and surfaces. Biointerfaces. 2006; 49:79-86.

18. Santos DKF, Rufino RD, Luna JM, Santos VA, Saruboo LA. Biosurfactants: multifunctional biomolecules of the 21st century. Int J Mol Sci. 2016; 17(3):401. https://doi.org/10.3390/ijms17030401.

19. Sarubbo LA. Production and stability studies of the bioemulsifier obtained from a strain of Candida glabrata UCP1002. J Biotechnol. 2006; 9:400-401.

20. Shahaby AF, Alharthi AA, Tarras AEE. Bioremediation of petroleum oil by potential biosurfactant-producing bacteria using gravimetric assay. Int J Curr Microbiol Appl Sci. 2015; 4(5):390-403.