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# CHARACTERIZATION OF BROWNFIELD: EX-SITU DETECTION OF HYDROCARBON DEGRADING AND BIOSURFACTANTS PRODUCING MICROFLORA

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Global efforts of forestation and reutilization of barren lands to mitigate climate change have increased in last decade. Brownfields are non-utilizable barren lands due to the presence or potential presence of hazardous substance, pollutant, or contaminant. In present study, brownfield of Chak Naurang, Punjab, Pakistan contaminated with petroleum hydrocarbon was analyzed in order to evaluate the possibility of bioremediation. Physicochemical analysis of composite soil sample demonstrated that contaminated soil was slightly alkaline clay-loam with TPH of 22.2 g/kg. The concentration of nitrogen, phosphorus and potassium was found to be quite low i.e. 644 mg/kg, 12.46 mg/kg and, 20 mg/kg, respectively. Electrical conductivity (E.C) i.e., 4.3 dS/m and 18% water content showed the deteriorated condition of affected soil. The crude oil degrading and biosurfactants producing microorganisms were isolated from the contaminated soil using soil enrichment technique. The isolated microorganisms were tested for biosurfactants production on the basis of qualitative and quantitative assays. Our results showed that efficient biosurfactants producing microorganisms belong to the genera of *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Achromobacter*, *Meyerozyma* and *Aspergillus*. Statistical analyses exhibited significant differences ($p<0.05$) amongst the biosurfactants production capabilities of the characterized strains. These findings revealed that contamination of petroleum hydrocarbons has adversely effected soil composition and agriculture properties. The affected soil harbors diverse microbial communities that can produce biosurfactants and use crude oil as sole carbon substrate. Moreover, nutrients amendments could be an effective strategy to stimulate bioremediation process and for improving soil productivity.
**Keywords:** Brownfields, bioremediation biosurfactants, microorganisms, agriculture.

## INTRODUCTION

Brownfields are defined as the abandoned, underused and polarized industrial sites, which are characterized by the presence of toxic pollutants in high concentration (Mahzouni, 2017). Petroleum contaminated brownfields are becoming an important issue for public, environmental agencies and local governments. Revitalization of these sites is of utmost importance to generate significant socio-economic benefits (Martinat *et al.,* 2016). In Pakistan, most of the crude oil contaminated brownfields are unproductive for decades because of their incompatibility for agriculture or commercial use. In Punjab, province of Pakistan, these brownfields are mostly located in proximity of the suburban or rural agriculture settings posing a serious threat to the public health (Mohanram *et al.,* 2016). Crude oil is a complex and heterogeneous mixture of a variety of hydrocarbon. Considering the chemical heterogeneity and associated toxicities, petroleum hydrocarbons are enlisted as priority pollutants by EPA (LeBlanc, 2016). In soil system, different constituents of crude oil, specifically poly aromatics hydrocarbons, bind strongly with soil particles and damage its agro-physical properties (Fatima *et al.,* 2015). Conventional approaches for remediation of contaminated brownfields include excavation, dig and dump, landfilling, transport, incineration, the addition of oxidants ($KMnO_4$ or $H_2O_2$) and soil washing (Rizzo *et al.,* 2016). However, due to cost ineffectiveness of these treatment methodologies, most of the brownfields have been left abandoned in Pakistan. Bioremediation technologies could be very effective and eco-friendly for the revitalization of these contaminated brownfields. The successful field operation of bioremediation relies mainly on the presence of adequate nutrients and hydrocarbon degrading microbial communities that can produce surface active compounds (Gudiña *et al.,* 2015). Biosurfactants are extracellular microbial products that show a high degree of structural, functional and chemical heterogeneity. They are naturally secreted by hydrocarbon degrading microorganisms under nutritionally stressed conditions (Mnif and Ghribi, 2015). In comparison with conventional chemical surfactants, biosurfactants exhibit environmental compatibility, biodegradability, high reaction rate, better physio-chemical characters and synthesis of peculiar micro structures in polar and non-polar environments. These molecules interplay at the interfaces of different systems through the formation of micro-emulsions

and cause solubilization of hydrophobic compounds to improve their bioavailability for the microbial cells (Perfumo *et al.,* 2010; Zhu *et al.,* 2014).

Many previous findings have demonstrated that hydrocarbons contaminated brownfields harbor diverse biosurfactant producing microorganisms and could help restoration of the soil for agriculture activities. But the key limiting factor of natural soil remediation of contaminated soils is nutrient deficiency. Therefore, status of the nutrients and presence of crude oil degrading microbes at brownfields hold paramount importance for successful revitalization of the site. In this work, soil nutrients assessment and associated parameters were determined in order to explore the possibility of site rehabilitation using bioremediation. In addition, presence of hydrocarbons degrading microbial communities and their biosurfactants producing abilities were also investigated.

## MATERIALS AND METHODS

***Sampling site and soil characterization*:** The oil field of Chak Naurang with the coordinates 32°59'46"N and 72°56'41"E was selected as sampling site. Samples contained contaminated soil from brownfield along with natural/unaffected soil as control (Fig. 1). The contaminated soil was collected from three locations at a depth of about 10-30 cm, labeled, and placed aseptically in sterile zipper plastic bags. Samples were air dried and passed through a mesh sieve to remove stones and debris. Soil texture was determined using hydrometer method (Kettler *et al.,* 2001), pH and Electrical Conductivity-E.C (dS/m) through a digital pH and conductivity meter, water content (%) by the oven drying method, Total Petroleum Hydrocarbons-TPH (g/kg) with the help of gravimetric method, Total Organic Carbon-TOC (g/kg) through the heat colorimetric method of potassium dichromate dilution (Wang *et al.,* 2013), total Nitrogen-N (mg/kg) by applying Kjeldahl method (Sainju, 2017), available Phosphorous-P (mg/kg) as extractable phosphorus using sodium bicarbonate ($NaHCO_3$) (Uddin *et al.*, 2012), and available Potassium-K (mg/kg) using suitable extractants and flame photometer (Behera *et al.*, 2011). All the readings were taken in triplicates.

***Culture conditions*:** One gram of contaminated soil sample was inoculated in 100 ml minimal salt medium (MSM) containing 10 g/l of $K_2HPO_4$, 5 g/l of $NaH_2PO_4$, 2 g/l of $NaNO_3$, 0.2 g/l of $MgSO_4.7H_2O$, 0.01 g/l of $CaCl_2.2H_2O$, 0.08 g/l of $FeSO_4.7H_2O$ and 1% (v/v) crude oil as sole carbon source. Culture medium was incubated in 250 ml Erlenmeyer flask at 37°C and 150 rpm for a total of 30 days for enrichment. After a period of every 5 days, 1 ml of inoculum was transferred into freshly prepared MSM containing 1% (v/v) crude oil and re-incubated for next 5 days. During all cycles of enrichment, pH of the medium was adjusted to 7.0.

***Isolation crude oil degraders*:** Post enrichment, serial dilution method was used for isolation of microorganisms.

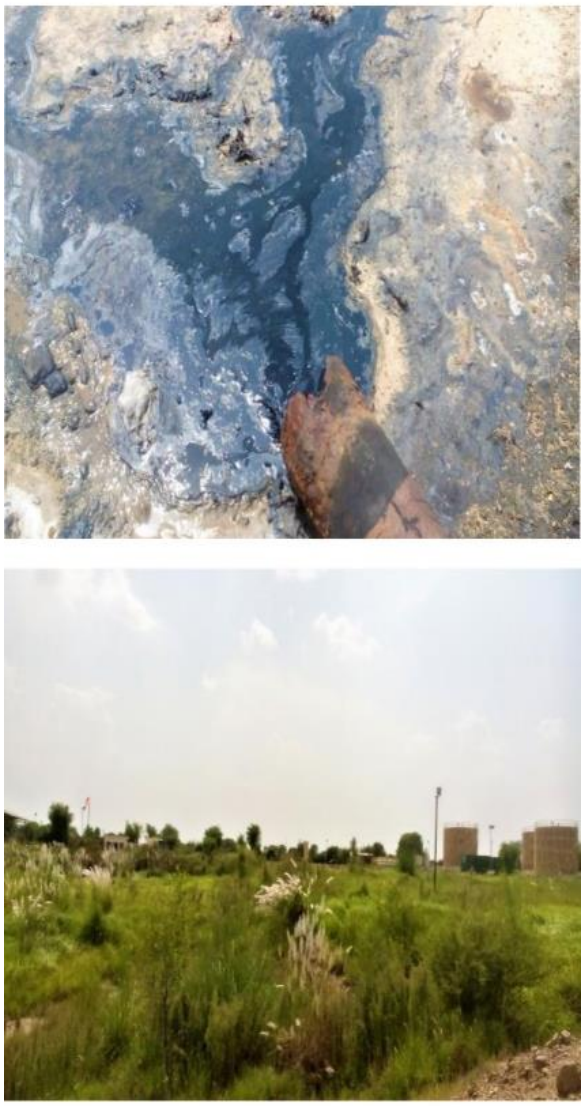

**Figure 1. Different regions of crude oil affected Brownfield and the unaffected agricultural land of Chak Naurang**

The inoculum was spread on Nutrient Agar plates and Sabouraud Dextrose Agar plates followed by incubation at 37 and 25°C, respectively, for a time period of 24-96 h to obtain discreet bacterial and fungal colonies. Replica Plate Method was used for further purification of colonies.

***Qualitative Screening of Biosurfactant Producing Microorganisms*:**

***Blood agar hemolysis assay*:** Individual microbial colonies were inoculated using spot inoculation method on blood agar plates containing 5% fresh human blood. Visual inspection of plates was done after 24-96 h and the hemolytic activity was designated as α (alpha), β (beta) or γ (gamma) corresponding to the zones of hemolysis (Varjani *et al.,* 2014).

***CTAB methylene blue agar plate assay*:** This assay involves the bonding of cationic bromide salt present in the medium with the extracellularly secreted anionic surfactants that forms an insoluble complex, visible due to methylene blue. For screening and detection of glycolipids or other negatively charged surfactants, post inoculation and successive incubation of plates, the aforementioned complex appeared around colonies of biosurfactant producers in the form of a blue halo. Zones of clearance were observed and measured (Walter *et al.,* 2010).

***Crude oil overlay agar assay*:** Crude oil-coated Mueller Hinton Agar plates were inoculated with colonies of isolated strains and incubated at 37°C for 24–96 h. Results were recorded after observing diameter of an emulsified area around the site of inoculation indicating the presence of surface active compounds (Shoeb *et al.,* 2015).

***Identification of microorganisms*:** Qualitative screening assays resulted in the selection of potential bacterial, fungal and yeast isolates which were identified using morphological, biochemical and phylogenetic tools. Morphological identification included colony morphology and Gram staining of bacterial species whereas Lactophenol Blue staining and observation of characteristic growth patterns of fungal hyphae. Biochemical characterization of bacterial strains was accomplished by following standard protocols stated in Bergey's Manual of Systematic Bacteriology. The yeast strain was identified through a distinctive glossy appearance of cell colony, Gram positive characteristics and microscopy. Molecular identification was done through sequencing of conserved 16S and 18S rRNA regions of the extracted genome at Macrogen Sequencing Centre, Korea.

***Quantitative Analyses of Biosurfactant Producing Microorganisms*:**

***Emulsification index (E.I $_{24}$) assay*:** Culture supernatant retrieved from identified strains was studied for emulsification properties using kerosene oil as standard (Walter *et al.,* 2010). The test tube containing equi-volume of filtrate and kerosene oil was vortexed for 1 minute and placed at room temperature for 24 h. $E.I_{24}$ is calculated by using the following formula.

$$E.I_{24} = \frac{\text{Height of emulsion layer (cm)}}{\text{Total Height of the liquid column (cm)}} \times 100$$

***Surface tension measurement*:** Surface tension was measured using plate method to enumerate the production capability of isolates. Easy Dyne K20, KRÜSS GmbH Tensiometer (Germany) was set on surface tension mode and readings were confirmed thrice (Marajan *et al.,* 2015).

***Oil displacement assay*:** 20 ml of distilled water was taken in a petri plate with 20 µl of crude oil on top, followed by addition of supernatant (20 µl) over the oil film. After 30 seconds, a zone of displacement was witnessed and measured accordingly (Youssef *et al.,* 2004).

***Statistical analysis*:** Biosurfactant quantitative assays were performed in triplicates for which means, and standard errors were calculated. One-way analysis of variance was used to determine significant ($p<0.05$) variation within results. Tukey's-HSD test was applied to evaluate multiple comparisons between the means. The shape of distribution, central value, and variability in data was represented using box plots.

## RESULTS

***Soil characterization*:** Physicochemical properties of soil samples collected from Chak Naurang brownfield and the unaffected site (control) have been summarized in Figure 2. It was observed that the affected soil was clay-loam in texture with slightly alkaline pH. 46% of moisture content was detected in the agricultural soil which significantly decreased up to 18% in the affected soil. The crude oil contaminated soil showed less electrical conductivity (4.3 dS/m) as compared to the agricultural soil (24.81 dS/m). However, considerably high quantity of TPH (up to 22.56 g/kg) and TOC (23.11 g/kg) was detected in the contaminated soil since the recorded value of TOC for agricultural soil was 4.03 g/kg. Contrary to foretasted parameters, reduced concentration of nitrogen (0.644 g/kg), phosphorous (12.46 mg/kg) and potassium (20 mg/kg) was detected in the affected brownfield.

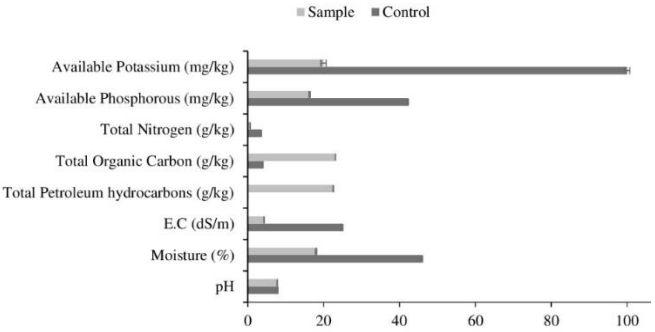


**Figure 2. Comparative analysis of physicochemical properties of crude oil affected and unaffected soil**

***Isolation of microorganisms*:** Post soil characterization and enrichment of thirty days, isolation resulted in a total of thirty-four bacterial, five fungal and one yeast strain from the hydrocarbon contaminated soil. Isolates were purified and codded as RB, RF, and RY for representation of bacterial, fungal and yeast species, respectively.

***Qualitative screening of biosurfactant producing microorganisms*:** Post 72-96 h of incubation, 45% of strains showed α-hemolysis, 37 % of strains showed β- hemolysis and 18% of strains showed no hemolytic activity on Blood Agar plates. For CTAB Methylene Blue Agar assay, 32% of strains produced a dark blue halo around the point of inoculation, 38% of strains produced a comparatively lighter zone whereas the remaining 30% of strains displayed no zone. Crude Oil Overlay Agar assay indicated that 65% of strains

were hydrocarbon degraders (Fig. 3). These findings helped in the selection of potential strains for identification studies and quantitative estimation of biosurfactants production.

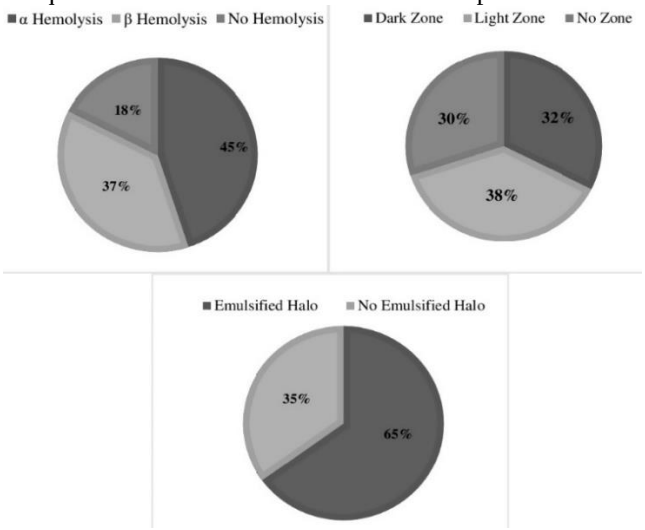


**Figure 3. Qualitative screening of biosurfactants producing microorganisms**

***Identification of biosurfactant producing microorganisms*:** Identified strains exhibited phylogenetic diversity because of their homology with various bacterial, yeast and fungal genera. Morphological and biochemical tests showed that most of the bacterial isolates were positive for Gram staining, catalase, citrate and, motility. Yeast species was Gram positive whereas, for fungal isolates, spores were visible in characteristic hyphae post-Lactophenol Blue staining. Molecular identification was accomplished through amplification and sequencing of conserved 16S and 18S rRNA regions followed by comparison with the database of known rRNA sequences and submission at the National Centre for Biotechnical Information (NCBI). GenBank IDs of strains along with characteristic properties and closest relatives have been presented in Table 1 and Figure 4.

***Quantitative Analyses of Biosurfactant Producing Microorganisms*:**

***Emulsification index (E.I $_{24}$) assay*:** *Pseudomonas aeruginosa* (MF069166) turned out to be the highest emulsifier with 84% $E.I_{24}$ followed by *Meyerozyma* sp. (MF138126) which resulted in 82% emulsification of kerosene oil. Besides these strains, *Pseudomonas* sp. (MF099829), *Bacillus amyloliquefaciens* (MF138127), *Bacillus thuringensis* (MF138122), *Bacillus* sp. (MF138130), *Pseudomonas stutzeri* (MF138118), *Aspergillus tereus* (MF138128) and *Bacillus licheniformis* (MF138121) were also found to be good emulsifiers with $E.I_{24}$ values of 79, 77, 75, 72, 70 and 69%, respectively. Box plots were constructed for emulsification index to display data variation (Fig. 5). A significant difference of less than 0.001 was found amongst the emulsification properties of strains (Table 2).

**Table 1. Identification of biosurfactants producing microorganisms.**

| Isolates | Gram Staining | Species as close relatives | Homology (%) | Accession Number |
|---|---|---|---|---|
| RB 3 | + | *Bacillus pumilus* | 97.0 | MF138116 |
| RB 5 | + | *Paenibacillus azoreducens* | 98.0 | MF159559 |
| RB 6 | - | *Pseudomonas stutzeri* | 98.0 | MF138118 |
| RB 7 | + | *Bacillus licheniformis* | 98.0 | MF138121 |
| RB 9 | + | *Bacillus thuringiensis* | 99.0 | MF138122 |
| RB 11 | - | *Achromobacter xylosoxidans* | 95.0 | MF138123 |
| RB 12 | + | *Bacillus cereus* | 96.0 | MF138124 |
| RB 27 | - | *Pseudomonas aeruginosa* | 98.0 | MF069166 |
| RB 29 | + | *Bacillus subtilis* | 98.0 | MF138125 |
| RB 31 | + | *Bacillus* sp. | 98.0 | MF138130 |
| RB 32 | + | *Bacillus amyloliquefaciens* | 97.0 | MF138127 |
| RB 33 | - | *Pseudomonas* sp. | 99.0 | MF099829 |
| RY36 | + | *Meyerozyma* sp. | 99.0 | MF138126 |
| RF38 | --- | *Aspergillus terreus* | 98.0 | MF138128 |

**Table 2. Analysis of Variance for Emulsification index, Surface tension and Oil displacement assays**

| Emulsification Index (E.I $_{24}$) | CODE | Df | Sum Square | Mean Square | F value | Pr(>F) |
|---|---|---|---|---|---|---|
| | | 13 | 961 | 73.9 | 112 | <0.001 |
| | Residuals | 28 | 19 | 0.7 | | |
| Surface Tension (S.T) | CODE | 13 | 896 | 68.9 | 379 | <0.001 |
| | Residuals | 28 | 5 | 0.2 | | |
| Oil Displacement | CODE | 13 | 76.3 | 58.7 | 648 | <0.000 |
| Assay (O.D.A) | Residuals | 28 | 0.3 | 0.01 | | |


**Figure 4. Phylogenetic characterization of potential biosurfactants producing microorganisms**

***Surface tension measurement*:** A substantial decrease in surface tension confirmed the presence of surface active compounds in the extracted supernatant of isolates. *Meyerozyma* sp. (MF138126) was efficient with S.T value of 25 mN/m whereas, *B. amyloliquefaciens* (MF138127) reduced the S.T up to 39.8 mN/m. Other strains like *P. aeruginosa* (MF069166), *P. azoreducens* (MF159559) and *P. stutzeri* (MF138118) exhibited notable S.T values of 26.6, 33.7 and 34.3 mN/m as shown in Figure 6. One-way ANOVA showed statistical significance whereas, Tukey's HSD revealed pairwise differences amongst means ($p< 0.05$) (Table 3).

***Oil displacement assay*:** *P. aeruginosa* (MF069166) displaced the thin film of crude oil up to 8 cm whereas *B. pumilus* (MF138116) formed a zone of 3.5 cm. The remaining strains displayed remarkable surface active properties by

forming displacement zones from 3.9 cm to 7.4 cm (Fig. 7). The statistical hypothesis was accepted due to significant differences in results of ODA. *p*-value was less than 0.05 and data variation was confirmed through Tukey's test (Table 4).

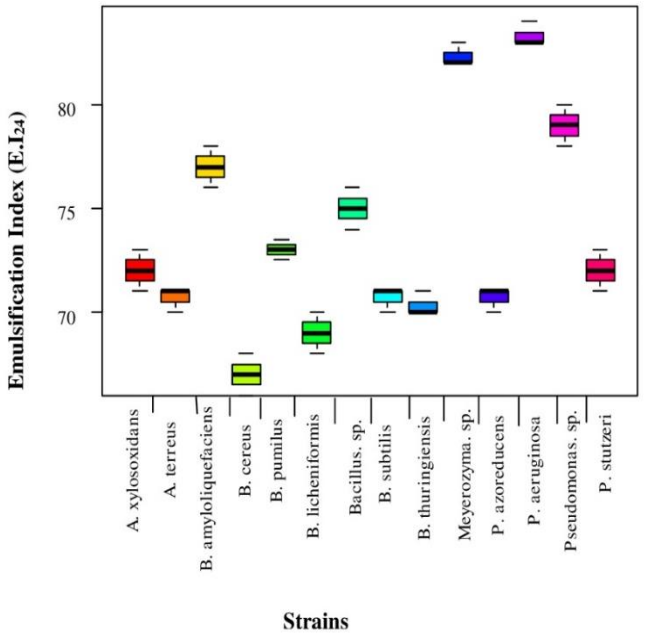


**Figure 5. Emulsification Index (E.I$_{24}$) of potential biosurfactants producing microorganisms**

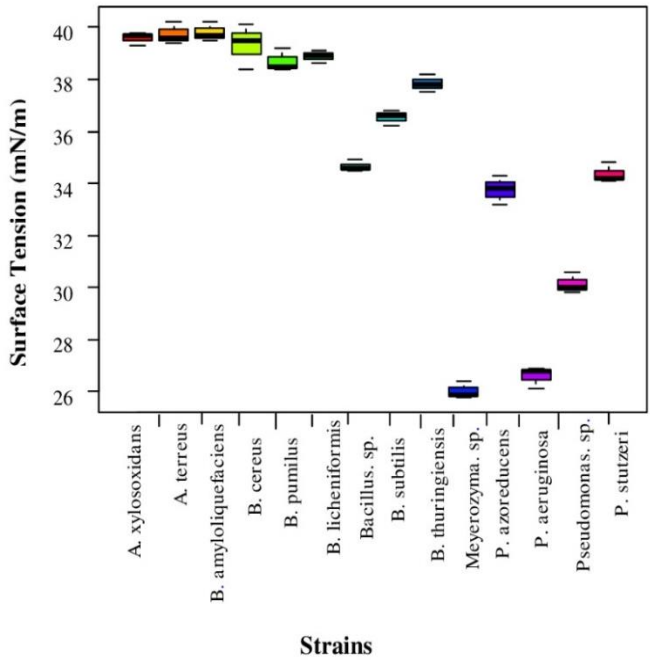


**Figure 6. Surface Tension (S.T) of potential biosurfactants producing microorganisms**

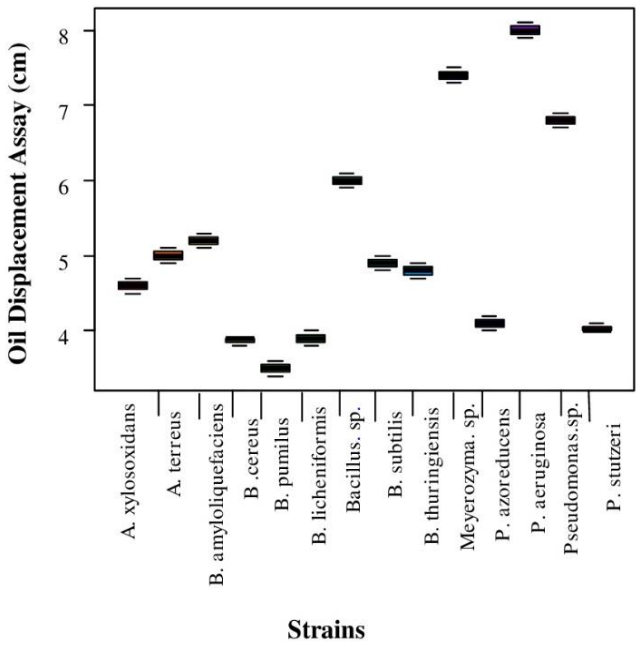


**Figure 7. Oil Displacement Assay (O.D.A) of potential biosurfactants producing microorganisms**

## DISCUSSION

Brownfield revitalization has become the subject of public and government interests because of their potential in economic and sustainable regional development (Strom, 2018). The decision making process for the selection of suitable revitalization technology has been quite challenging and based on various site specific conditions including geochemistry of the site, nature and concentration of the contaminant and presence of appropriate microflora that could possibly help in the mitigation of pollutant (Trellu *et al.,* 2016). Current investigation was focused on the characterization of the petroleum contaminated brownfield and detection of biosurfactants producing and petroleum degrading microflora in order to access the feasibility of bioremediation for site revitalization. The results of soil analysis revealed that the site was heavily contaminated with crude oil hydrocarbons up to 22.66 g/kg. The high level of petroleum hydrocarbons and very low concentration of nutrients (N, P, K) are considered as major limiting factors for site remediation. In addition, very less vegetation cover was observed at the site that could be directly associated with toxicity of hydrocarbons and scarcity of nutrients in the soil. Likewise, some previous studies have reported very low vegetation at the hydrocarbons contaminated soil (Moubasher *et al.,* 2015; Fatima *et al.,* 2016). Substantial decrease in E.C of contaminated sample was observed due to the non-polar nature of crude oil that hinders the free movement of ions in soil and makes it more hydrophobic (Vincent *et al.,* 2011).

Saturated hydraulic activity, clogged pore spaces, reduced water permeability or increased bulk density due to hydrophobic coating of oil on clay particles might be responsible for reduced water content in petroleum affected areas (Khamehchiyan *et al.,* 2007). Collectively, these conditions imply that petroleum hydrocarbon pollution has caused deterioration in soil physiochemical and biological properties. Therefore, application of inorganic NPK fertilizer should be included in the remedial measures to restore soil nutrients ratios and stimulate soil biological activities.

The hydrophobicity of the crude oil constituents poses a serious challenge in mass transfer of the contaminants and makes them more persistent (Lacatusu *et al.,* 2017). The hydrocarbon contaminated environments harbor diverse microbial communities that have the ability to degrade the contaminant and use them as sole source of carbon and energy. Recently, Vargas *et al.* (2017) reported twenty-four and Yadav *et al.* (2016) reported nineteen biosurfactant producers from different oil spill affected areas. These microbes produce extracellular biosurfactants in order to emulsify the hydrocarbons and increases bioavailability of the contaminants for their intracellular degradation. In present study, fourteen crude oil degrading and biosurfactants producing microorganisms were characterized on the basis of qualitative and quantitative assays. Blood agar hemolysis assay has been recommended as one of the primary screening assays for the identification of biosurfactants producing microorganisms by many researchers, however, due to high possibility of giving both false negative and false positive results, this assay should be supported by other methods for verification (Elazzazy *et al*., 2015; Sharma *et al.,* 2015). Majority of the microorganisms forming blue halos around their respective colonies suggested the production of anionic biosurfactants in CTAB Methylene Blue Assay (Pacwa-Płociniczak *et al.,* 2016). In crude oil overlay assay, distinct emulsification zones were observed around the microbial colonies due to biosurfactants production and degradation of hydrocarbons. (Kokare *et al.,* 2007) stated the efficacy of crude oil overlay agar assay for simultaneous detection of biosurfactants and hydrocarbon degradation. In contrast to many previous findings, the present research work demonstrates that not even a single isolate was found to be negative for any qualitative plate assay (Ali *et al.,* 2013; Santhini, 2014; El-Gamal *et al*., 2015). Amongst the isolates, promising biosurfactant producing microorganisms were molecularly identified that belonged to the genera of *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Achromobacter*, *Aspergillus* and a recently established one, *Meyerozyma*. Some of the characterized species like *P. aeruginosa* (MF069166), *Meyerozyma* sp. (MF138126), *Pseudomonas* sp. (MF099829), *P. azoreducens* (MF159559), *B. amyloliquefaciens* (MF138127), *B. thuringensis* (MF138122), *Bacillus* sp. (MF138130), *P. stutzeri* (MF138118), *A. tereus* (MF138128), and *B. licheniformis* (MF138121) expressed remarkable phenotypes of biosurfactant production. Prevalence of *Pseudomonas* and *Bacillus* species at crude oil contaminated sites and their role as eminent biosurfactant producers have been described previously (Mnif *et al.,* 2011; Bezza and Chirwa, 2015; Deng *et al.,* 2016). However, the presence of some new species like *Meyerozyma* sp. and *Paenibacillus azoreducens* tempts us to propose that hydrocarbons contaminated brownfields can be quite productive for exploration of novel biosurfactant producing microorganisms. For quantitative estimation of biosurfactants, methods like oil displacement assay, emulsification index analysis and surface tension measurement were preferred as they provide more reliable, efficient and consistent results (Al-Bahry *et al*., 2013; Ahmad *et al.,* 2016). All the characterized isolates showed excellent surface active properties and a positive correlation was observed between emulsification and surface activity of biosurfactants. Statistical variations in quantitative assays showed that every isolate differs in its potential to produce biosurfactants. This variability in results could be attributed to the diverse chemical nature of biosurfactants produced by different microorganisms (Ismail *et al.,* 2015). Taking everything, crude oil affected brownfield of Chak Naurang showed excellent potential for bioremediation because of the surfactants producing and oil degrading microbial diversity. In addition, the nutrients amendment could be very useful for the restoration of soil physiochemical and biological properties in order to create opportunities from these unproductive lands.

***Conclusions*:** The results of present study focus on the characterisation of abandoned brownfield of Chak Naurang, Punjab, Pakistan and to evaluate feasibility of bioremediation. Soil analysis revealed high TPH, low N, P, K concentrations, decreased EC and low water content in the crude oil affected brownfield. Moreover, the contaminated soil harbours crude oil degrading and surfactants producing microflora at the site. The application of nutrients (NPK) could be very effective to facilitate microbial growth and biodegradation activity for brownfield revitalisation.

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