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Isolation and Production of Biosurfactant by Bacteria Isolated from Oil Polluted Soil

Mohammed Inuwa Ja'afaru*, Joyce Briska and Aishatu Addawuro Salihu

Department of Microbiology, Modibbo Adama University of Technology, Yola, Nigeria.

* Corresponding author: Mohammed Inuwa Ja'afaru; e-mail: mijaafaru@mautech.edu.ng

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ABSTRACT

A total of 45 bacteria were isolated from automechanic workshops. All the isolates were screened for biosurfactant production using oil spreading test, blood haemolysis test and the drop-collapse test. Three of the isolates that showed capacity to produce biosurfactants in the screening test were identified as *Bacillus subtilis* A3, *Pseudomonas aeruginosa* C3 and *Alcaligenes faecalis* by microscopic and biochemical analysis. The isolate produced biosurfactants during growth on diesel containing medium. Biosurfactant produced by *Bacillus subtilis A3* reduced the surface tension of water to 29.85 mN/M, compared to surface tension of 24.02 mN/M produced by sodium dodecyl sulphate. The emulsification index of the 3 isolates was lower than that of sodium deodecyl sulphate (SDS). The E_{24} of the biosurfactant from the 3 bacteria were stable at 100 ^{o}C and between pH 2 – 12. In the soil washing experiments, higher amounts of oil were recovered from the oil contaminated soil using the bacteria produced biosurfactant than with SDS. The findings indicate that the biosurfactant produced by the isolates could be useful in bioremediation applications.

Keywords: Biosurfactant, *Bacillus*, *Alcaligenes*, *Pseudomonas*, Oil pollution.

1. INTRODUCTION

The exploitation, transportation, consumption, spill and disposal of petroleum and its products often leads to serious environmental problems [1]. Soil pollution with petroleum products has become one of the main concerns due to its content of chemical and hazardous materials. Many techniques have been discovered and studied for soil treatment and one of the most applicable methods is soil washing by surfactants [2].

Biosurfactants are amphiphilic biological compounds produced extracellularly or as part of the cell membrane by a variety of yeast, bacteria and filamentous fungi [3]. The hydrophilic moiety of biosurfactants can be carbohydrate, amino acid, phosphate group or some other compounds, whereas the hydrophobic moiety is a long chain fatty acid [4]. They have several advantages over the synthetic surfactant such as lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, high selectivity, and specific activity at extreme pH, temperature, salinity and the ability to synthesize from renewable feedstock [5]. These unique properties allow the use of biosurfactant in a great number of industrial applications such as in oil recovery and in emerging technologies like microbial remediation of hydrocarbon and crude oil contaminated soil which is one of the problems of petroleum waste management. Large quantity of dehydrated oil sludge generated in the disposal of oil-contaminated sewage needs to be rendered harmless to human and to the environment.

Due to the potential risk or hazard to environment and human health due to the discharge of residual synthetic surfactants in soils and groundwater, an improved strategy for soil washing technology is to use biosurfactants. The main difficulty in bioremediation of oil-contaminated soil is the bioavailability of the oil pollutants in soil, causing poor food-microorganism contact and thus poor biodegradation efficiency. As a result of their hydrophobicity, oil hydrocarbons bind consistently to soil particles (thus reducing the

bioavailability of oil compounds to microorganism), forming holes (in soil) that may exclude water and nutrients [6]. The key process to enhance the bioavailability of the oil contaminant is to transport the pollutant to the aqueous bulk phase [7]. One way to increase the bioavailability of hydrophobic pollutants in soil is using surfactants to enhance the mobility and solubilization of petroleum hydrocarbons, thereby facilitating their degradation by microorganisms [8]. Examples of some bacteria that produce biosurfactants include *Bacillus, Acinetobacter, Micrococcus, Mycobacterium, Pseudomonas, Xanthomonas and Klebsiella* [9].

The aim of this research is to investigate the use of biosurfactant produced by some bacteria to remove crude oil from laboratory contaminated sand.

2. MATERIALS AND METHODS

2.1 Sampling and Isolation of bacteria from soil sample

Oil polluted soil was collected from different automobile workshops at different locations within Jimeta-Yola metropolis of Adamawa state**.** Isolation of bacteria was achieved by inoculation of serially diluted soil samples (10^{-3}) onto nutrient agar (supplemented with 1% (v/v) crude oil) plates. Individual colonies of bacteria which varied in shape and color were picked and purified by repeated sub-culturing on nutrient agar. The isolates were screened for biosurfactant producing ability by the following tests.

2.2 Blood haemolysis test

A fresh colony from the plate was picked and streaked on blood agar plates containing 5% (v/v) human blood. The plates were incubated at 37^0 C for 48 hours. Haemolytic activity was detected by the presence of a clear zone around the colony. Observation was made for α and β haemolysis. The clear zone indicated the presence of biosurfactant producing organisms [10].

2.3 Oil displacement test: The oil spreading assay was carried out as developed by Morikawa *et al.* [11]. Forty ml of distilled water was poured into a petri dish and 2 ml of crude oil was gently added unto the water to form a thin oil layer. One ml of culture broth of the test organism was gently placed at the centre of the oil layer. The presence of biosurfactant in the supernatant was indicated by the formation of clearing zone around the oil layer. The diameter of the clearing zone was recorded in centimeter (cm).

2.4 Drop collapse assay

For the drop collapsed assay, 0.2 ml mineral oil was added into each well of a 96 well micro titer plate. The plate was equilibrated for 1 hour at room temperature and then 0.2 ml of the culture broth of the test organism was added to the surface of the oil. The shape of the drop on the oil surface was inspected after 1 minute. Broth giving flat drops was scored as positive i.e. biosurfactant producers and those cultures that gave rounded or beaded drops were scored as negative, which is indicative of lack of biosurfactant production. Water was used as negative control while sodium dodecyl sulphate was used as positive control [10].

2.5 Biosurfactant Production

One ml of diesel oil was added to 100 ml of mineral salt medium of Jacobucci *et al*. [12] containing (g/l) 1.8 K_2HPO_4, 1.2 KH_2PO_4, 2.0 NH_4NO_3, 0.2 $MgSO_4.7H_2O$, 0.1 NaCl and 0.01 $FeSO_4.7H_2O$ in a conical flask and the pH adjusted to 7 using sodium hydroxide. The medium was sterilized by autoclaving at 121^0C for 15 minutes and allowed to cool before being inoculated with 1 ml of broth culture of the selected bacterial isolates that were positive during screening. The inoculated flasks were incubated at room temperature for a period of 7 days, with shaking at regular time interval.

Biosurfactant was extracted from the medium after cell removal by centrifugation at 4000 rpm for 20 minutes. The pH of the supernatant was adjusted to 2 using 1M H_2SO_4. 75 ml mixture of chloroform/methanol (2:1 v/v) was added to 100 ml of MSM containing the grown isolates and vigorously shaken for several minutes and allowed to set. The mixture was left to stand overnight for evaporation. After 24 hours, the organic layer was transferred to a round bottom flask and the aqueous layer was re-extracted two times for complete recovery of the biosurfactant. The organic phases were combined yielding viscous brown-colored sediment which was considered as the crude biosurfactant [13].

2.6 Surface tension and Emulsification activity (E_{24}) measurements

The emulsification index was measured by adding 2 ml of mineral oil to 2 ml of cell-free extract of biosurfactant and vortexing at high speed for 2 minutes. The mixture was left to stand for 24 hours at room temperature and the readings were taken. Sodium dodecyl sulphate (SDS) was used as positive control. The E_{24} was determined as

$$= \frac{Height\ of\ emulsion\ layer}{Height\ of\ liquid\ layer} \times 100 \quad [1].$$

The surface tension the medium was determined using the capillary tube method and SDS was used as positive control. The surface tension of the cell-free broth was calculated using the formula,

$$as = \gamma = \frac{gphr}{2}$$

Emulsification stability studies as describe by [14] were carried out using cell free broth obtained by centrifugation process.

2.7 Effect of temperature

Two ml of cell-free broth was measured and exposed to different temperatures (4^0C, 27^0C and 100^0C) for 30 minutes. The emulsification index was determined as described above.

2.8 Effect of pH

To study the effect of pH on the biosurfactant, the pH of the cell free-broth was adjusted to 2, 7 and 12 using HCl and sodium hydroxide respectively. The emulsification index was then measured.

2.9 Removal of oil by Biosurfactant through kinetic assay

The removal of crude oil from the laboratory contaminated soil was tested through the saturation of 50 g of soil with 10% of crude oil . The laboratory contaminated soils were placed in 250 ml Erlenmeyer flasks, to which 50 ml of the crude biosurfactant (cell-free broth after fermentation) were added. The Erlenmeyer flasks were shaken at 200 rpm for 24 h at 28 ^{o}C. The entire content was then centrifuged at 5000 rpm for 1200 s. Following the washing of the soil, the samples were treated with 50 ml hexane twice for the removal of residual oil. The amount of oil residing in the sand after the impact of biosurfactant was gravimetrically determined as the amount of residual oil after extraction with hexane and the % of oil removal was calculated using the equation:

$$\frac{Amount\ of\ residual\ crude\ oil}{Amount\ of\ crude\ oil\ added\ in\ the\ media}\ x\ 100$$

[7]

3. RESULTS AND DISCUSSION

3.1 Isolation of Biosurfactant Producing Organisms

Forty five bacteria were isolated from oil polluted soil. The isolates were tested for their ability to produce biosurfactant by blood haemolysis, drop collapse and oil displacement activity and the result recorded as seen in Table 1. These three methods were used by [15]. Results of the blood haemolysis test showed that nineteen out of the 45 isolates were α haemolytic, eighteen β haemolytic and eight isolates showed no hemolytic activity on blood agar plate. Fifteen (15) isolates out of the 45 gave flat drop within 5 minutes in the drop collapse test and were scored as positive. All the 15 isolates that were positive for drop collapse test were also positive for the oil displacement activity, as was also reported by [16]. The diameter of clearing around the oil layer ranged from 1.7 cm to 3.1 cm.

Biochemical characterization of the 15 biosurfactant producing organisms showed that they were *Bacillus subtilis* (5), *Bacillus circulans* (1), *Bacillus coagulans* (1), *Pseudomonas aeruginosa* (6) and *Alcaligenes faecalis* (2).

Based on the diameter of the clearing zone on oil displacement, three isolates i.e. *Bacillus subtilis* A3, *Pseudomonas aeruginosa* C3 and *Alcaligenes faecalis* E1 were selected and used for biosurfactant production.

Table 1: Screening of Isolates for Biosurfactant Production.

S. No	Isolate code	Heamolytic activity	Drop collapse	Oil displacement (cm)
1	A1	B	+	2.6
2	A2	A	+	2.4
3	A3	A	+	3.0
4	A4	B	+	1.8
5	A5	A	-	-
6	A6	A	-	-
7	A7	-	-	-
8	A8	-	-	-
9	A9	A	-	-
10	B1	A	+	2.8
11	B2	B	+	2.5
12	B3	A	+	1.9
13	B4	B	+	2.1
14	B5	-	-	-
15	B6	B	-	-
16	B7	-	-	-
17	B8	β	-	-
18	B9	A	-	-
19	C1	B	+	2.8
20	C2	A	+	2.4
21	C3	B	+	3.1
22	C4	A	-	-
23	C5	B	-	-
24	C6	B	-	-
25	C7	A	-	-
26	C8	B	-	-
27	C9	-	-	-
28	D1	A	+	1.9
29	D2	A	+	1.8
30	D3	B	-	-
31	D4	-	-	-

32	D5	B	-	-
33	D6	B	-	-
34	D7	-	-	-
35	D8	B	-	-
36	D9	A	-	-
37	E1	A	+	2.4
38	E2	B	+	1.7
39	E3	-	-	-
40	E4	A	-	-
41	E5	B	-	-
42	E6	B	-	-
43	E7	A	-	-
44	E8	-	-	-
45	E9	A	-	-

KEY: α = alpha heamolysis, β = beta heamolysis, + = positive, - = negative

Table 2: Surface Tension and Emulsification Activity of Biosurfactant Activity Produced by Bacteria.

Isolates	ST (mN/m)	E_{24} (%)
Bacillus subtilis A3	29.85	66.4
Pseudomonas aeruginosa C3	30.40	65.0
Alcaligenes faecalis E1	31.31	64.5
Sodium dodecyl sulphate	24.02	68.4

KEY: ST: Surface Tension, E_{24}: Emulsification Activity

3.2 Determination of Biosurfactant Activities

Table 2 shows the result of surface tension measurement (ST) and emulsification activity (EA) of the crude biosurfactant produced by *Bacillus subtilis* A3, *Pseudomonas aeruginosa* C3 and *Alcaligenes faecalis* E1.

The emulsification activity of Sodium Dodecyl Sulphate (1 mg/ml) was measured as 68.4% while E_{24} of 66.4%, 65% and 64.5% was measured for biosurfactant produced by *Bacillus subtilis* A3, *Pseudomonas aeruginosa* C3 and *Alcaligenes faecalis* E1 (1 mg/ml) respectively. The emulsification activity of commercial surfactant is thus slightly higher than biosurfactant produced by the bacteria. Similar results were also reported by [16], [1] and [17]. This shows that biosurfactant can increase the bioavailability of crude oil and biodegradation process.

The ability of the crude biosurfactant from the 3 test bacteria and SDS to reduce surface tension of water were compared. SDS reduced the surface tension of water to a minimum value (24.02 mN/m) in this study, followed by *Bacillus subtilis* A3 biosurfactant (29.85 mN/m). Biosurfactant produced by *Alcaligenes faecalis* E1 showed the least reduction in surface tension (31.31 mN/m).

Estimation of the biosurfactant activity showed that biosurfactant produced by the isolates considerably reduced the surface tension of medium from 70 to 29.85 mN/m by *B. subtilis,* 30.40 mN/m by *Pseudomonas aeroginosa* and 31.31 mN/m by *Alcaligenes faecalis.* To evaluate the effect of environmental condition on performance of crude biosurfactants produce by the test organisms, the thermal and pH stability were studied.

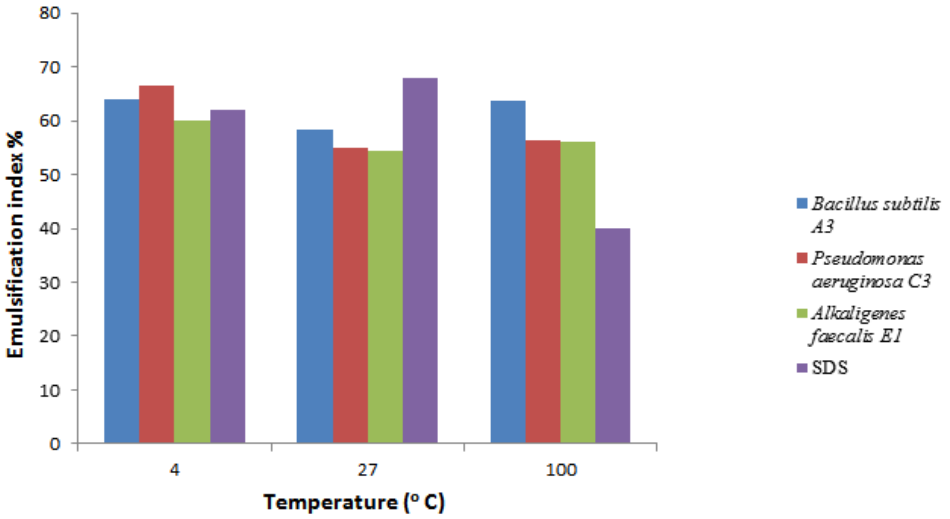

Figure 1: Effect of Temperature on Emulsification Activity of Biosurfactants Produced by Bacteria.

3.3 Effect of temperature

The effects of temperature on the stability of the biosurfactant produced by the bacterial isolates shows that E_{24} were found to be stable (>50%) at all temperatures tested (Figure 1). This is in agreement with the work reported by [18] that biosurfactant produced by *Bacillus subtilis* was stable at temperatures of 4, 27 and 100 ^{o}C. Aparna *et al*. [1] reported biosurfactant produced by *Pseudomonas aeruginosa* to be stable over wide range of temperature (4-100^{0}C). The stability of biosurfactant produced by *Alcaligenes faecalis* was also as reported by [19]. On the contrary, loss of activity was observed by the synthetic surfactant at high temperature (>50^{0}C). This agrees with the work of [15] who observed loss of activity at temperature above 70^{0}C.

3.4 Effect of pH

The effects of pH on the stability of biosurfactant produced by the bacterial isolates shows no appreciable change was observed at the pH range of 2-12 (Figure 2). This agrees with the work reported by [20] where no changes were observed at the pH ranges of 2-12 when biosurfactant produced by *Bacillus subtilis* was used. When compared to the synthetic surfactant, there was loss of activity at pH 2. The results show that biosurfactant produced by the selected isolates meets requirements involving extreme pH and temperature of any oil reservoir [9].

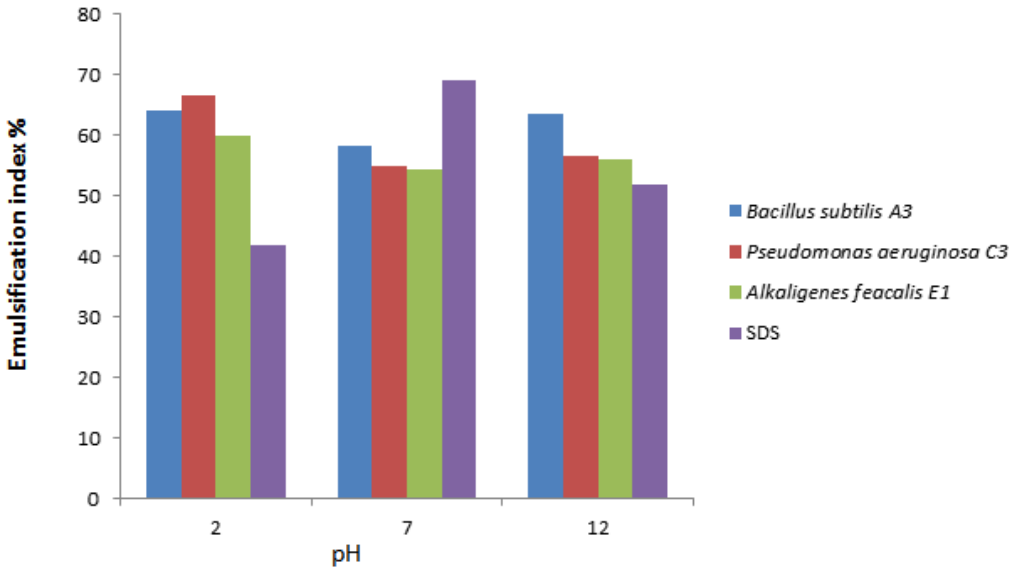

Figure 2: Effect of pH on the Emulsification Activity of Biosurfactants Produced by Bacteria.

Table 3: Crude Oil Recovery (%) by Crude Biosurfactant Produced by Bacteria.

Biosurfactant producing Isolates	Crude oil Recovered (%)
Bacillus subtilis A3	48.80
Pseudomonas aeruginosa C3	46.04
Alcaligenes faecalis E1	40.30
Sodium dodecyl sulphate	40.20
Water	20.04

3.5 Crude Oil Recovery

Table 3 shows the result of sludge washing experiment. Biosurfactant produced by the test isolates were used to elute crude oil from the oily sludge compared with SDS and water. Biosurfactant produced by *Bacillus subtilis* A3 was able to recover 48.8% of oil in sludge, *Pseudomonas aeruginosa* C3 recovered 46.04% and *Alcaligenes faecalis* E1 recover 40.30% of oil in sludge.

Similarly, oil recovering from the sludge showed that the SDS was able to recover 40.20% while distilled water showed the lowest recovery of 20.04%. The result of oil recovery from oil sludge by the biosurfactant produced by the selected isolates compared with SDS and distilled water (Table 3) shows that biosurfactant produced by the three isolate had a better recovery compared to the synthetic surfactant and water. Surfactant produced by *Bacillus subtilis* recover up to 48.80% of oil in sludge, followed by *Pseudomonas aeruginosa* produced surfactant that recovered 46.04%, and *Alcaligenes faecalis* produced surfactant recovered 40.30% of crude oil in sludge. Similarly the percentage recovered by SDS was 40.20% while distilled water showed the least crude oil recovery of 20.04%. The result agrees with the work reported by [21] where biosurfactant produced by *Pseudomonas aeruginosa* was used to recover crude oil from an oily sludge and percentage recovered was 41.10% while biosurfactant produced by *Bacillus*

subtilis recovered up to 48.51% as reported by Zheng *et al*. [16].

4. CONCLUSION

In conclusion, biosurfactant produced by *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Alcaligenes faecalis* showed good emulsification activity with high stability and good surface active properties. It was found to be stable over wide range of pH and temperature thus making it suitable for various industrial applications. Attempt to recover crude oil from the sludge under laboratory was successful with biosurfactants.

5. REFERENCES

1. Aparna A, Srinikethan G, Smitha H (2011). Effect of addition of biosurfactant produced by Pseudomonas spp on biodegradation of crude oil. J Environ Sci Technol 6: 71-75.
2. Khodadadi A, Ganjidoust H, Razavi, SNS (2012). Treatment of crude-oil contaminated oil using biosurfactants. J Petrol Gas Engg 3:92-98.
3. Pacwa-Plociniczak M, Plaza GA, Piotrowska-Saget Z, et al (2011). Environmental application of biosurfactants: a review. Intern J Mol Sci 12:633-654
4. Al-Araji L, Abdul Rahman RNZR, Basri M, et al (2007). Microbial Surfactant - Miniriview. Asia Pac J Mol Bio Biotech 15: 99-105.
5. Desai JD, Banat IM (I997). Microbial production of surfactant and their commercial potential. Microb Mol Biol 61: 47- 64.
6. Pacheco, GJ, Ciapina EMP, Gomes E de Barros, et al (2010). Biosurfactant production by Rhodococcus erythropolis and its application to oil removal. Braz J Microbiol 41: 685-693.
7. Chaprao MJ, Ferreira INS, Correa PF, et al (2015). Application of bacterial and yeast biosurfactants for enhanced removal and biodegradation of motor oil from contaminated sand. Elect J Biotechnol 18: 471-479.
8. Lai CC, Huang YC, Wei YH, et al (2009). Biosurfactant-enhanced removal of total petroleum hydrocarbons from contaminated soil. J Hazard Mat 167:609-614.
9. Rosenberg E, Ron E Z (1999). High and low molecular mass microbial surfactants. Appl Microbiol Biotechnol 52: 154-162.
10. Youssef NH, Duncan KE, Nagle DP, et al (2004). Comparison of methods to detect biosurfactant production by diverse microorganisms Microbiol Methods 56: 339-347.
11. Morikawa M, Hirata Y, Imanaka T (2000). A study on the structure-function relationship of lipopeptide biosurfactants. Biochem Biophy Acta 1488: 211-218.
12. Jacobucci DFC, Vasconcelos CK, Matsuura A, et al (2001). Degradation of diesel oil by biosurfactant producing bacterial strains. The Mag of Environ Assess Remed 8: 3-10.
13. Helmy Q, Kardena E, Nurachman Z, et al (2010). Application of biosurfactant produced by Azotobacter vinelandii AV01 for enhanced oil recovery and biodegradation of oil sludge. Intern J Civ Engng 10: 7-14
14. Sarubbo LA, Farias CBB, Campos-Takaki GM (2007). Co-utilization of canola oil and glucose on the production of a surfactant by Candida lipolytica. Curr Microbiol 54: 68-73.
15. Zheng C, Wang M, Wang Y, et al (2012).Optimization of biosurfactant-mediated oil extraction from oil sludge. Biores Technol 110: 338-342
16. Miller-Maier R, Bodour AA (1998). Application of a modified drop collapse technique for surfactant quantification and screening of biosurfactant producing microorganisms. J Microbiol Meth 32: 273¬280.
17. Hossein S, Saleh M (2009). Microbial enhanced oil recovery using biosurfactant produced by Alcaligenes faecalis. J Biotech 7: 216
18. Phitnaree S, Vatchtarin R, Furu Sako K, Aran H (2008). Production and application of biosurfactant from Bacillus subtilis muv4. J Sci Technol 30: 87-93.
19. Joshi S, Bharucha C, Jha S, et al (2008) Biosurfactant production using molasses and whey under thermophillic . Biores Technol 99:195-199.
20. Cipinyte V, Saulius G, Dovile S, et al (2011). Production of biosurfactant by Arthrobacter sp. N3, A hydrocarbon degrading bacterium. Environ Technol Res 1:68-75.
21. Lima TMS, Fonseca AF, Leao BA, et al (2011). Oil recovery from fuel oil storage tank sludge using biosurfactant. Biorem Biodegrad 2:1-5.

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