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Microbial Studies of Biosurfactant Producing Bacteria from Crude Oil Contaminated Soil

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ABSTRACT: Soils contaminated with crude oil collected from three sites in Delta State were analyzed using serial dilution and pour plate methods. The physicochemical characteristics of the soil were determined using standard methods. The mean heterotrophic bacteria count and the mean hydrocarbon utilizing bacteria count were also determined. Blood haemolysis, foaming activity, oil spreading techniques and emulsification assay was used to screen the microorganisms for biosurfactant production. The biosurfactant producing bacteria were selected and their effect on metal removal and growth kinetics was also determined. The pH of the contaminated soil samples ranged 4.82 to 5.62. The mineral elements such as potassium (k^+) and sodium (Na^+) ranged from 0.20 to 0.80 Meq/100g and 0.07 to 0.81 Meq/100g respectively. The heavy metal content such as zinc (Zn^{2+}) and lead (Pb^{2+}) ranged from 10.13 to 19.24 mg/kg and 19.24 to 49.63 mg/kg respectively. Organic carbon and THC ranged from 5.44 to 6.87 % and 2720.00 to 3110.00 mg/kg respectively. The mean heterotrophic bacteria and hydrocarbon utilizing bacteria ranges from $1.4 \pm 0.4 \times 10^6$ to $1.6 \pm 0.7 \times 10^6$ and $1.0 \pm 0.2 \times 10^6$ to $1.2 \pm 0.4 \times 10^6$ respectively in the soil samples. Bacteria isolated and identified from the three sites include *Corynebacterium* spp., *Bacillus subtilis*., *Pseudomonas aeruginosa*, *Micrococcus varians*, *Streptococcus* spp., *Klebsiella* spp. and *Enterobacter* spp. with *Bacillus subtilis* and *Pseudomonas aeruginosa* having the highest prevalence and among these isolates only *Corynebacterium* spp., *Bacillus subtilis*., *Pseudomonas aeruginosa* and *Micrococcus varians* showed positive result for the production of biosurfactant. *Bacillus subtilis* had the highest metal removal capacity of 29.44% of 100 ppm of lead. *Corynebacterium* spp. and *Bacillus subtilis* had an optimum growth at pH 8 and 7 respectively while *Pseudomonas aeruginosa* and *Micrococcus varians* has an optimum growth at pH

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Biosurfactant are surface active molecule that are mainly synthesized extracullarly and as part of cell membrane by certain microorganisms which are amphyphilic in nature, that is, having hydrophilic (water loving) and hydrophobic (exclude water) moleties which confers them with the capacity to exist between phases such as water/air or water/oil, thus helps to minimize the surface and interfacial tension of the surface and interface respectively (Banat 1995; Karanth *et al.,* 1999). Biosurfactant microorganisms make use of organic compound as carbon and energy source for growth. When these carbon and energy sources are in insoluble form such as hydrocarbon, they either make the influx of hydrocarbon possible by producing biosurfactant or the nature of the cell wall is modified and this is done via production of nomionic or lipopolysaccharide surfactant in their cell wall (Guerra *et al.,* 1984; Hauser and Karnovesky, 1954; Guerra *et al.,* 1986). The fate of petroleum hydrocarbon in the soil depend on the biodiversity of biosurfactant as well as hydrocarbon utilizing microorganisms in the contaminated soil in that numerous indigenous microorganisms such as bacteria, fungi, actinomycetes are capable of using hydrocarbon as source of carbon and energy for growth. Also, the major factor influencing the biodegradation of crude oil pollutant in the environment is their non-availability to microorganisms. Thus, ways to activate these potentials must be considered in that most degradation potentials are widely distrusted among microorganisms and also indigenous microbes are usually present in a very small amount. Hydrocarbon varies in their susceptibility to microbial attack and their biodegradability in soil is determined by various factors such as nutrient, oxygen, pH, composition, concentration and bioavailability of the contaminant as well as the soils physicochemical characteristics. Synthetic surfactant cannot be used in the cleanup of soils polluted with crude oil in that it has very negative and detrimental effect on humans as well as the environment. Bioremediation requires large amount of biosurfactant microorganism but there are problem of low yield and high production cost, thus reducing their

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industrial application. Microbial degradation of soil polluted with crude oil is the major natural means of remediating crude oil contaminated environment (Akpoveta *et al*., 2011) and more biosurfactant producing microorganisms should be identified, studied and employed in remediation process as diverse groups of bacteria and fungi have been isolated from soils polluted with crude oil and shown to produce biosurfactant and degrade hydrocarbon in the laboratory.

MATERIALS AND METHODS

Sample Collection: Soil sample was collected from crude oil contaminated soil from three sites, Batan Community, Odidi community and Kantu community, all in Warri-Southwest Local Government Area Delta State. This was done with the help of a clean hand trowel at about 5 cm depth, which is the top soil where the concentration of the contaminant is expected to be high. The samples were kept in a clean plastic polyethylene bag and were transported to the laboratory for analysis (Karthik *et al*., 2010).

Physicochemical analysis of crude oil contaminated soil: The pH reading was obtained with the aid of a Hanna microprocessor pH multimeter (Ekaise and Nkwelle, 2011) Determination of Na K Mg Ca was determined using a flame photometer according to methods of Onyeonwu, (2000). Minerals (heavy) metals analyses for determination of Zinc (Zn), Iron (Fe), Mercury (Hg), Lead (Pb) and Copper (Cu), were done by aspirating the solution for (analysed) each metal analysis into the Atomic Absorption Spectrometer (ASS) PG 550 model (Adelekan and Abegunde, 2011). Determination of Nitrate, Sulphate and total organic carbon was done using methods described by Onyeonwu, (2000). THC was extracted using n – hexane (Akpoveta *et al*., 2011).

Isolation and Enumeration of Microorganism: Ten (10) gram of the samples was weighed into sterile beaker and 90 ml of sterile distilled water was added. This was thoroughly mixed and serial diluted up to 10^{-6} using 10 fold dilutions. One (1) ml of the dilution was plated in nutrient agar for bacteria isolation and in potato dextrose agar for fungi isolation, both in duplicates. The nutrient agar plates were incubated at 35ºC for 48 hours while the potato dextrose was incubated at room temperature. After incubation, the number of discrete colony on the plate was counted in terms of Cfu/g using 0.1ml inoculums size.

*Enumeration and Isolation of Hydrocarbon Utilizing Bacteria:*Bacteria with hydrocarbon degrading ability were isolated from the contaminated soil samples using mineral salt agar that is added with15 g of agar and about 1 % crude oil as carbon source (Amund *et al*., 1987), with a final pH of 7.0. One (1) ml of 10 mg/l Nystsatin was applied to the plate to prevent Fungi growth. The culture plates were then incubated at 35ºC for 7 days. All colonies seen was counted and expressed in colony forming unit per gram at the end of the incubation period.

Production of Biosurfactant: The selected samples were inoculated in a 250 ml conical flask having 50 ml of sterile mineral salt medium with 2 ml petrol added as carbon source. The broths were amended with Nystatin to prevent contamination by fungi. A reciprocal shaker was used to incubate the culture broth at 120 rpm for 5 – 7 days at 30ºC (Mulligan, 2005).

Extraction of Biosurfactants: A 50ml R2B broth was used to culture the inoculums to which 1ml of petrol was added and was incubated at 25ºC for 7 days with shaking conditions. The cultures were centrifuged at 5000 rpm, 4ºC for 30 minutes after incubation to remove the bacteria cells. 1M H_2SO_4 was added to the supernatant to adjust the pH at 2 with equal volumes of chloroform methanol added in the ratio of 2:1. These mixtures were properly mixed and were left over night for evaporation. The presence of biosurfactant is indicated by a white colored precipitate at the interface of the liquids.

Screening of the Isolates for Biosurfactant Production: Pure cultures of the isolated colonies was obtained and tested for their biosurfactant production by the following methods.

Blood Haemolysis Test: Each Isolated pure culture was streaked on Blood Agar Plate and was incubated for 49 Hours at 37ºC. The presence of clear zone around the colonies was observed (Anandaraj and Thivakaran, 2010).

Foaming Activity: Each pure isolates was grown separately in a 100 ml conical flask, each containing 50 µl of nutrient broth medium. After incubation at 31ºC for 48 hours, it was placed on a shaker for 24 hours and a forming activity if seen is an indication of a positive result (Banant, 1995).

Oil Spreading Techniques (Oil Displacement Activity): To 40ml distilled water in a Petri dish, 10 µl of crude oil was added to the surface to form a thin oil layer. Then 10 µl of culture or culture supernatant was gently placed on the centre of the oil layer. The displacement and formation of a clear zone as an indication of a positive result and the diameter of the clear zone was measured after 30 seconds. The clear

zone is measured in biosurfactant unit which is the amount of surfactant forming 1 cm^3 of oil displaced area (Banant, 1995).

Emulsification Assay: According to Cooper and Goldenberg, 1987. 2 ml of petrol was collected into a test tube containing 2 ml of the culture supernatant after centrifugation and was vortexed at a very high speed for 5 minute to make sure of homogenous mixture of both liquid. The activity of emulsification was observed after 12 minutes and was calculated by,

$$\mathrm{EI\ (mm)} = \frac{\mathrm{Tota\ Height\ og\ EL}}{\mathrm{Total\ Height\ of\ LL}}$$

Where EI = Emulsification index (mm); EL = Emulsified Layer; LL = Liquid Layer

The Effect of Biosurfactant Producing Microorganism on Metal Removal: The isolates was grown in nutrient broth at a neutral pH in the presence of 100 ppm of lead and are incubated for 5 five days (37°C, 120 rpm) to allow the interaction of the organisms with the metal ions. The samples were withdrawn at 24 hours interval and the concentration of the metal ions left in solution was examined by AAS. Uninoculated flask containing the metal was used as control (Patil *et al.,* 2012).

Growth Kinetics of Isolated Strains: The selected isolates were inoculated in 50 ml mineral salt medium that was adjusted to a pH of 6, 7 and 8 for bacteria isolates using sodium hydroxide and H_2SO_4. This was kept in a shaker at 25 °C for 144 hours at in absorbance 600 nm, reading was taken at regular interval of 12 hours (Dahalan *et al.,* 2014).

RESULTS AND DISCUSSION

The physicochemical result given in (Table 1) shows that the soil samples are acidic with pH ranging from 4.82 to 5.62. Organic carbon and THC of crude oil contaminated soils ranged from 5.44 to 5.87 and 2720 to 3110 mg/kg respectively. Iron, zinc and copper content in contaminated soil ranges from 0.10 to 0.42 mg/kg, 1.42 to 2.17 mg/kg and 0.05 to 0.45 mg/kg for copper. Lead ranged 0.54 to 1.38 in contaminated soil. Table 2 shows the mean total heterotrophic and hydrocarbon utilizing count in cfu/g of the soil samples in which Batan community had the highest mean heterotrophic and hydrocarbon utilizing bacteria count of $1.6\pm0.7\times10^6$ and $1.2\pm0.4\times10^6$ respectively while Odidi community having the least of $1.4\pm0.4\times10^6$ and $1.0\pm0.2\times10^6$ respectively. Figure 1 shows the prevalence of bacteria isolates. *Bacillus subtilis* and *Pseudomonas aeruginosa* had the highest prevalence of 100%. Table 3 shows the result for blood hemolysis test and foaming activity. *Corynebacterium, Bacillus subtilis, Pseudomonas aeruginosa and Micrococcus varians* showed positive result by the formation of a clear zone around the colonies. *Corynebacterium* spp., *Bacillus substilis*, *Pseudomonas aeruginosa* and *Micrococcus varians* also showed good foaming activity. Table 4 shows the result of oil spreading technique with *Corynebacterium* spp., *Bacillus substilis*, *Pseudomonas aeruginosa* and *Micrococcus varians* showing positive results with *Pseudomonas aeruginosa* having the highest diameter width of 5.2 cm and *Corynebacterium* spp. having the least width of 2.5 cm.

Table 1: Physicochemical properties of Contaminated and Uncontaminated soil sample

Soil Parameters	Unit	Batan	Odidi	Kantu	Uncontaminated Soil
pH		5.62	5.42	4.82	6.90
K^+	mg/kg	0.80	0.20	0.71	10.04
Na^+	mg/kg	0.78	0.07	0.81	10.03
Mg^{2+}	mg/kg	0.84	0.54	0.42	10.09
Ca^+	mg/kg	4.86	3.65	4.89	12.05
SO_4^{2-}	mg/kg	5.88	4.32	2.50	11.20
NO_3^-	mg/kg	4.05	5.64	3.81	20.40
Fe^{2+}	mg/kg	0.42	0.38	0.10	0.01
Zn^{2+}	mg/kg	2.17	1.58	1.42	0.34
Cu^{2+}	mg/kg	0.09	0.05	0.45	0.01
Pb^{2+}	mg/kg	1.42	0.54	1.38	0.09
Org Carbon	%	6.87	6.40	5.44	2.23
THC	Mg/kg	3110.00	2720.00	2984.00	105.12

Table 5 shows the emulsification index of the bacteria isolates with *Pseudomonas aeruginosa* showing the highest emulsification activity of 51 mm while *Corynebacterium* spp. had the least emulsification activity of 26 mm. *Corynebacterium* spp., *Bacillus substilis*, *Pseudomonas aeruginosa* and *Micrococcus varians* had metal removal capacity of 16.26%, 29.44%, 27.77% and 19.78% respectively. Figure 3 shows the growth kinetics of *Corynebacterium* spp., having maximum growth at pH 8 after 48 hours. The maximum growth was around 0.11 nm. Figure 4 shows the growth kinetics of *Bacillus subtilis*, having

the optimum growth at pH 6 and 7 after 36 hours. Figure 5 shows the growth kinetics of *Pseudomonas aeruginosa* having optimum growth at pH 8 after 36 hours. Figure 6 shows growth kinetics of *Micrococcus varians* having optimum growth at pH 8 after 60 hours.

This analysis shows that the pH of the contaminated soil samples were more acidic than the uncontaminated soil sample (Chukwuma *et al*. (2010). In all the soil samples, the concentration of potassium, sodium, magnesium, calcium, sulphate and nitrate was lower than those of the uncontaminated soil. However, the concentration of iron, zinc, copper and lead were higher in the contaminated soil sample than that of the uncontaminated soil which agrees with (Lloyd and Caskette, 2001) who reported a higher concentration of the element in top soil parts and roots of yellow lupine when the soil was polluted with diesel. Thus this high concentration of metal on crude oil contaminated soil may be as a result of the constant and continuous spillage of petroleum product on the soil which accumulated overtime.

Table 2**:** Mean bacteria load of crude oil contaminated soil samples

Locations	Total Heterotrophic Bacteria Count (Cfu/G)	Hydrocarbon Utilizing Bacteria Count (Cfu/G)
Batan community	1.6±0.7 x 10^6	1.2 ±0.4 x 10^6
Odidi community	1.4 ±0.4x 10^6	1.0 ±0.2x 10^6
Kantu community	1.5 ±0.6x 10^6	1.1± 0.3 x 10^6

Key: Values are in duplicate of mean ± standard deviation (S.D)

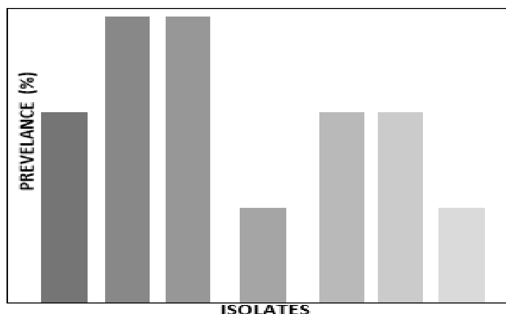

Fig 1: Prevalence of bacteria isolate in crude oil contaminated soils.

Table 3: Blood haemolysis test for bacteria strain

Bacteria Strain	Blood Haemolysis	Foaming Activity
Corynebacterium spp.	Positive	Positive
Bacillus subtilis	Positive	Positive
Pseudomonas aeruginosa	Positive	Positive
Micrococcus varians	Positive	Positive
Streptococcus spp.	Negative	Negative
Enterobacter spp.	Negative	Negative
Klebseillia spp.	Negative	Negative

Table 4: Oil Spreading Techniques of Bacteria isolates

Bacteria Isolates	Diameter (Cm)	Interpretation
Corynebacterium spp.	2.5	Positive
Bacillus spp.	5.0	Positive
Pseudomonas spp.	5.2	Positive
Micrococcus	5.0	Positive
Streptococcus spp.	Nil	Negative
Klebsiella spp.	Nil	Negative
Enterobacter spp.	Nil	Negative

Table 5: The emulsification index of bacteria strain:

Bacteria Isolates	Height Of Emulsion Layer (Mm)	Total Height Of Liquid Column (Mm)	Percentage emulsification index
Corynebacterium spp.	8	32	25
Bacillus subtilis	15	30	50
Pseudomonas aeruginosa	16	31	51
Micrococcus varians	12	30	40
Streptococcus spp.	Nil	Nil	Nil
Klebsiella spp.	Nil	Nil	Nil
Enterobacter spp.	Nil	Nil	Nil

The bacteriological analysis revealed a relatively high count in different soil samples. This high microbial load may be attributed to the existence of the normal flora of the soil and the ability of the isolated microorganisms to use petroleum product as sole carbon and energy source (Oluwafemi *et al.,* 2008). The isolates identified from the crude oil contaminated soil were *Corynebacterium* spp., *Bacillus substilis*, *Pseudomonas aeruginosa, Micrococcus varians*, *Streptococcus* spp., *Klebsiella* spp. and *Enterobacter* spp. (Adam *et al.*, 2014). These bacteria isolates have also been isolated from oil contaminated soil from Niger Delta aquatic system as hydrocarbon utilizing isolates as reported by (Ekaise and Nkwelle, 2011). The isolates *Corynebacterium* spp., *Bacillus substilis*, *Pseudomonas aeruginosa* and *Micrococcus varians*, showed positive result in all the five screening method used. Thus, it was observed that these isolates can produce biosurfactant. This is in accordance with the report of (Tabatabace *et al.,* 2005). Observation from haemolysis activity with the selected isolates showed positive result. This is in agreement with Nasr *et al.* (2009) who screen biosurfactant produced by *Bacillus substilis* and *Pseudomonas aeruginosa* using blood haemolysis test. Oil spreading method showed that the activity of the biosurfactant clearance zone on the soil surface correlate to the activity of the surfactant.

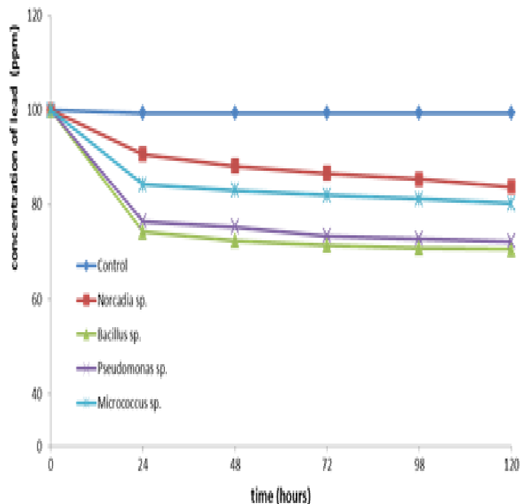

Fig 2: Heavy metal removal analysis of bacteria isolates by AAS (600nm)

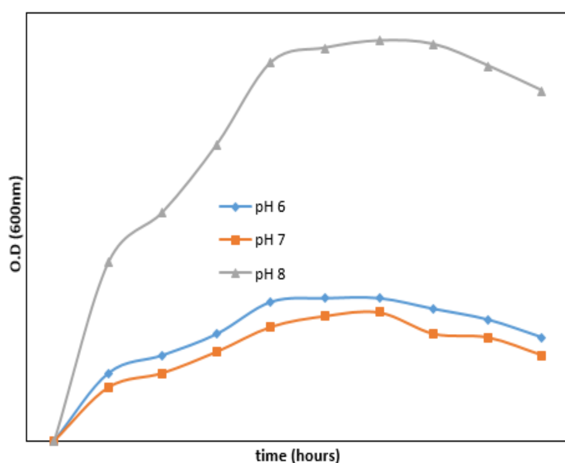

Fig 3: Growth kinetics of *Corynebacterium* spp., at pH 6, pH 7 and pH 8

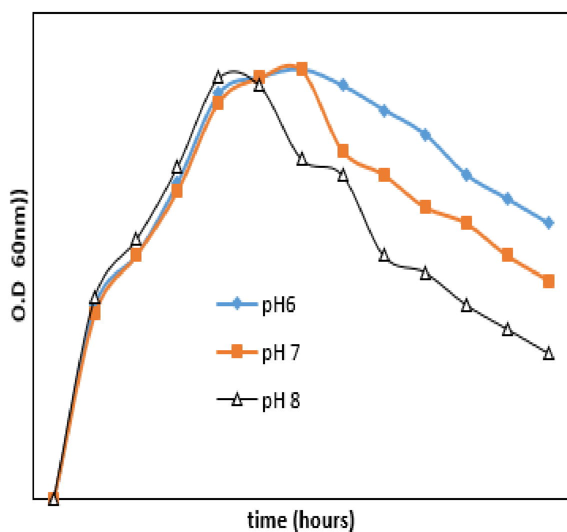

Fig 4: Growth kinetics of *Bacillus subtilis* at pH 6, 7, and 8

Previous report by Nasr *et al.* (2009) also shows biosurfactant activity by *Bacillus* spp. (19 mm), *Micrococcus* spp. (21 mm) *Pseudomonas* spp. (15 mm) which is similar with this study. Emulsification activity shows that *Pseudomonas* spp. was the best organism for biosurfactant production since the emulsification index was observed to be highest with *Pseudomonas* spp. This could be as a result of readily used nutrient in the broth medium. This finding is similar with those of Oluwafemi *et al*. (2008) who showed similar result with *Pseudomonas* spp. as good emulsifier and with emulsification index of 72%.

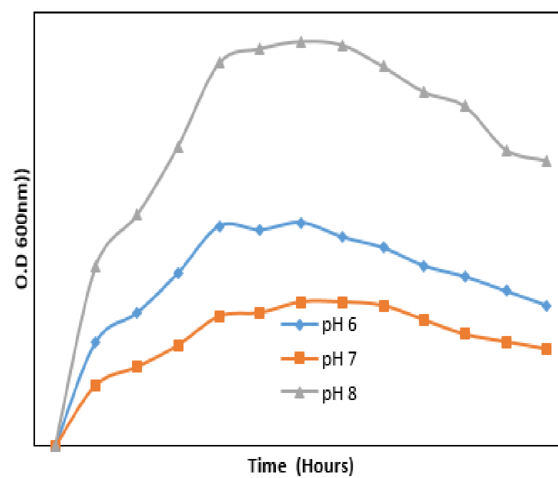

Figure 5: Growth kinetics of *Pseudomonas aeruginosa* at pH 6, 7 and 8

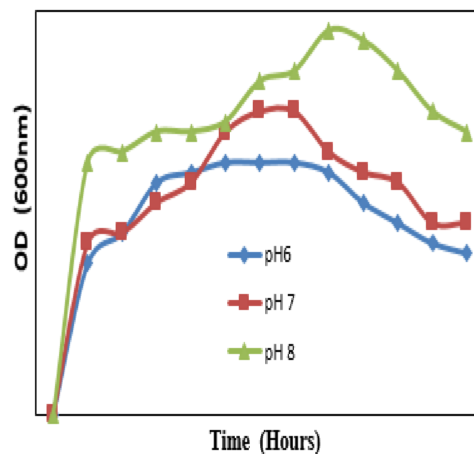

Fig 6: Growth kinetic for *Micrococcus varians* pH 6, 7, and 8

Among the various heavy metals contaminant, lead is known to be the leading toxicant worldwide and having various toxic effect on animal and human health as well as the environment. Thus there is need to remove these metals from the soil using microbes. Bacteria, fungi, yeast and algae can remove heavy metals and radio nucleotide from aqueous solution and in substantial amount as stated by (Yun and

Vijayaraghavan, 2008). They do not degrade heavy metals directly but can vange the valence state of metals into immobile or less toxic forms through biosurption of lead by functional group on the cell surface by complex formation and interaction between the acidic sites in the cell wall. This could be achieved via exclusion by intra and extracellular sequentiation, forming a permeable barrier, chelation and active transport enzymatic detoxification. This study reveals that the bacteria isolates *Bacillus subtilis, Pseudomonas aeruginosa*, *Corynebacterium* spp. and *Micrococcus varians* could remove about 16 – 30% metal from the media. This result is in agreement with Mulligan, (2005). This result also reveals that the isolates were tolerant to metal concentration of 100ppm. Thus resistance may be due to the ability of biosurfactant to complex with metal ions solution effectively (Atuanya and Osaghe, 2000).

Conclusion: This study has shown that *Corynebacterium* spp., *Bacillus substilis*, *Pseudomonas aeruginosa* and *Micrococcus varians* are capable of producing biosurfactant through systematic screening. These organisms are resistance to metals and are able to remove metals from crude oil contaminated soil. Based on their biodegradability and low toxicity, biosurfactant may have a promising future for use in cleaning up crude oil and heavy metal polluted soil.

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