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ISOLATION AND CHARACTERIZATION OF CRUDE OIL DEGRADING *BACILLUS* SPP.

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ABSTRACT

Today, application of microorganisms for removing crude oil pollution from contaminated sites as bioremediation studies, was considered by scientists because other methods such as surfactant washing and incineration lead to production of more toxic compounds and they are non-economic. Fifteen crude oil degrading *bacillus* spp. were isolated from contaminated sites. Two isolated showed best growth in liquid media with 1-3% (v/v) crude oil and mineral salt medium, then studied for enzymatic activities on tested media. The results showed maximal increase in optical densities and total viable count concomitant with decrease in pH on fifth day of experimental period for *bacillus S6*. Typical generation time on mineral salt with 1% crude oil is varying between 18-20h, 25-26h respectively for *bacillus S6* and *S35*. Total protein was monitored at determined time intervals as biodegradation indices. Increasing of protein concentration during the incubation period reveals that isolated bacillus can degrade crude oil and increase microbial biomass. These *bacillus* spp. reduced surface tension from 60 (mN/m) to 31 and 38 (mN/m), It means that these *bacillus* spp. can produce sufficient surfactant and have good potential of emulsification capacity. The results demonstrated that these *bacillus* spp. can utilize crude oil as a carbon and energy source.

Key words: Crude oil, *bacillus*, biodegradation, emulsification, surface tension, bioremediation

INTRODUCTION

Crude oil continues to be used as the principal source of energy and play an important role in the global environmental pollutant considerations. On the other hand, oil will remain as a major source of energy in the next several decades, because a reliable alternative energy comsumption has not yet been substituted. (Trindade *et al*., 2005, Al-Saleh and Obuekwe, 2005).

There are so many bacterial strains that can degrade or transform the components of crude oil products to the non-toxic, non hazardous, biodegradable and environmentally friendly compounds. This action is known as a biodegradation. The biodegradation of crude oil by microorganisms is one of the primary ways for eliminating crude oil from contaminated sites and appears to be the most environmentally friendly method of removal oil pollutant. (Korda *et al*., 1997; Kapley *et al*., 1999; Del Arco and De Franca, 2001; Barathi and Vasudevan, 2001).

Today use of microorganisms for removing crude oil pollution from contaminated sites as bioremediation was considered by scientists, because other methods such as surfactant washing and incineration lead to production of more toxic compounds and they are non-economic. (Margesin, 2000; Balba *et al*., 2002; Urum *et al*., 2003).

A wide range of studies have dealt with biotransformation, biodegradation, and bioremediation of petroleum hydrocarbons and interest in exploiting crude oil-degrading organisms for environmental clean-up has become central to petroleum microbiology. There are so many of bacterial and fungi with this ability (biodegradation of oil pollution) and these organism are widely distribute in marine, freshwater and soil habitats (head and Swannell, 1999), but scientist reported that indigenous and adapted microorganisms are more efficient for biodegradation of oil pollutant. The adapted organism degrades oil pollution normally but rate of this action is critically depends on different factors include microbial composition, contaminant type, geology of polluted site and chemical

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conditions at the contaminated site. There are different strategies for enhancing the rate of bioremediation of soil contaminated with crude oil include stimulation of the indigenous microorganisms, by introducing nutrients and oxygen into the soil (biostimulation), or through inoculation of an enriched mixed microbial consortium into the soil (bioaugmentation) (Barathi and Vasudevan, 2001; Seklemova *et al*., 2001).

As mentioned above, enrichment of degrading microbial communities and inoculation them into the contaminated site can be useful for removing oil pollutant from the environment (bioaugmentation) (Okoh, and Trejo-Hernandez, 2006).

In the present study, a number of *bacillus* species were isolated from contaminated site and some of their characterizations were determined such as the morphology, the capability to grow on crude oil and the ability to produce surfactant and hydrolytic enzymes.

MATERIALS AND METHODS

A brief description of the laboratory techniques used in this project is provided below.

Collection of soil samples

Previous studies reported the capability of native bacterial population to mineralize crude oil hydrocarbons in polluted soil and water, for this reason; in this investigation soil samples were gathered from crude oil contaminated sites (Okoh, 2003; Ojo, 2006).

Soil samples were obtained from contaminated area close to the storage and distribution center of oil products in Tehran refinery and Siri Island. All samples were collected in two replicates. Processing on soil samples began immediately upon arrival at the laboratory. The bacillus species indigenous in Crude oil contaminated soil samples were isolated by special techniques.

Isolation and characterization of crude oil–degrading bacillus spp.

1 g of each soil sample was dissolved in 9 mL of distilled water and heated at 80°C for 20 minutes. This thermal shock caused to all of vegetative microorganisms in soil sample died and spore of bacillus remained. The *bacillus* spp. was isolated by pour plate technique on plate count agar. Individual cultures were preliminary identified by morphological and biochemical techniques using the taxonomic scheme of Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). The obtained cultures of *bacillus* spp. were tested for hemolysis activity (Bicca *et al*., 1999) .Selected bacillus was examined for hydrocarbon utilization using crude oil as model substrate.

Growth of isolated bacillus on crude oil

Experiments were set up using 1-3% (v/v) of crude oil as a carbon source in basal mineral salt medium. The composition of the basal mineral salt medium used in this study was as follows (g/L):

$NaNO_3$(2.0 g/L), NaCl(0.8 g/L),KCl(0.8 g/L),$CaCl_2.2H_2O$(0.1 g/L), KH_2PO_4(2.0 g/L), $Na_2HPO_4.12\ H_2O$(2.0 g/L), $MgSO_4$(0.2 g/L), $FeSO_4.7H_2O$ (0.001 g/L);

2 mL trace element stock solution composed of (g/L): $FeCl_3.6H_2O$(0.08 g/L), $ZnSO_4.H_2O$(0.75 g/L), $COCl_2.6H_2O$(0.08 g/L), $CuSO_4.5H_2O$(0.075 g/L), $MgSO_4.\ H_2O$ (0.75 g/L), H_3BO_3(0.15 g/L), $Na_2MoO_4.\ 2H_2O$(0.05 g/L).

The initial pH was adjusted at 6.8. The medium was dispensed in 50 mL quantities into 250 mL erlenmeyer flasks. The flasks were cultivated in a gyratory shaker incubator programmed at 200 rpm and 30°C. Thereafter, the optical density (OD 600nm), total viable count (TVC), total protein (Bradford assay) and pH of the culture fluids were monitored at determined time intervals as biodegradation indices.

Crude oil degradation ability

Crude oil is a complex mixture of non-aqueous and hydrophobic components like n-alkanes , aromatics, resins, asphaltenes , and other organic compounds. Microorganisms degraded crude oil and used this source of carbon to produce cellular mass. Crude oil degradation ability was confirmed by cultivation of selected *bacillus* spp. on crude oil-mineral salt agar (1% v/v) and liquid medium with 1-3 % (v/v) crude oil and mineral salt medium at 30°. The appearance of the colonies on crude oil-mineral salt agar and increasing the turbidity of liquid media with 1-3 % (v/v) crude oil and mineral salt medium within the incubation period indicated that bacteria can growth on crude oil (Bola *et al*., 2006).

Colony counting procedures

The standard plate count technique was used to enumerate the microbial population each two day during the experimental period to reveal the number

of active bacteria in described medium. It is a direct quantitative measurement of the viable bacteria which are capable to grow on the selected medium. The samples were pipetted into the sterile petri dishes where a tempered agar medium has been added. The plates are rotated to evenly distribute the bacteria. Each colony that develops on or in the agar medium originates theoretically from one single bacteria cell. On the other hand the optical density of medium was measured daily to confirm the ability of bacteria to grow on the described medium.

Total protein estimation

For estimation of total protein, 1 mL of medium was taken and 9 mL water was added. It was centrifuged at 13000 rpm for 10 and boiled for 3 min. After cooling at room temperature, 1 mL of a $1MH_3PO_4$ solution was added. 50 µL aliquot was taken and mixed with 950 µL Coomassie protein assay reagent and incubated at 30°C for 10 min and the optical density was measured at 595 nm using UV-visible spectrophotometer. The total protein was estimated using a standard curve prepared with albumin (Bradford, 1976).

Emulsification test (E24)

This test was measured by using Cooper and Goldenberg method: 4 mL of Mineral Salt Medium after 48h incubation, and 6 mL hydrocarbon (oil) was added to each test tube. The mixture was vortexed at high speed for 2min, and then the test tubes were leaved to stand for 24h. The E24 index is given as percentage of height of emulsified layer (cm) divided by total height of the liquid column (cm) Cooper and Goldenberg (1999).

Hemolysis test and measurement of surface tension

Biosurfactant producing bacteria can decrease surface tension and increase mobility, bioavailability and subsequent biodegradation of crude oil. Biosurfactants are produced by many bacterial strains that can degrade or transform the crude oil. Decrease of surface tension that shown by tensiometer is known as a main scale of ability to producing surfactant (Abu Ruwaida *et al*., 1996). On the other hand biosurfactant producing bacteria have β hemolytic activity on Blood Agar medium and this test was used to decrease and eliminate inappropriate samples before using tensiometer. The isolated *bacillus* Cultivated on to Blood Agar and incubated at 30°C for 72h (Bicca *et al*., 1999). The bacteria with β hemolytic activity were selected, for measuring the surface tension. In this process 2.5 mL preculture of bacteria strains were prepared in NB. The preculture were added to 50 mL MSM and 1% filtered oil as hydrocarbon source. The mixtures with control samples were incubated at 30°C on shaker at 200 rpm for 2 days. The surface tension was measured by using a Du Neouy ring method. This method is based on the force needed to lift a platinum-iridium ring from the bulk of a sample fluid through the surface. All measurements were made at a temperature range of 23 – 25°C.

Enzyme activities testing

A little amount of old bacterial cultures (96h) on crude oil mineral salt agar was placed through a needle on the test media. The media were incubated at 28°C for 24-48h in the dark. Composition of media and detection of enzyme activities was done according to Parry *et al*., 2001.

RESULTS

Isolation and characterization of crude oil degrading bacillus spp.

Fifteen morphologically different *bacillus* colonies were isolated from contaminated sites. All isolates showed considerable growth on crude oil mineral salt agar (1%v/v). The colonies of these *bacillus* spp. were appeared within 48-72h on crude-oil-MSA. Two of them which showed best growth were selected (*S6* and *S35*).

Growth of isolated bacillus on crude oil

Utilization of crude oil as a substrate by isolated bacillus is shown by increasing the number of cells. Observations of both turbidity and viable cell count during the experimental period indicate that isolated *bacillus* can utilize crude oil as carbon substrate (Figs. 1-3). The generation time was 18 and 25h respectively for *bacillus S6* and *S35*. In the case of *bacillus S6* the maximum cell count was 5.7 log 10 cfu/mL in fifth day of experiment on 1 % (v/v) crude oil, but for the bacillus *S35* the maximum cell count obtained on sixth day (4.8 log 10 cfu/mL).

Total protein estimation

The protein estimation was effective method for monitoring the microbial population on the experimental medium. Increasing of protein concentration during the incubation period reveals

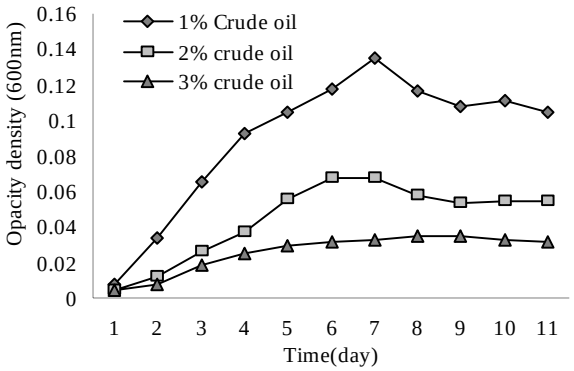

Fig. 1: Growth curve of *bacillus S35* on crude oil

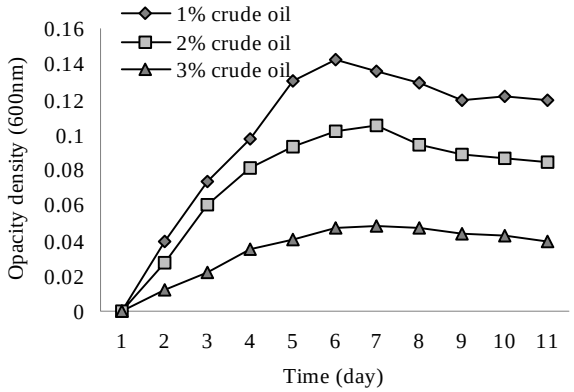

Fig. 2: Growth curve of *bacillus S6* on crude oil

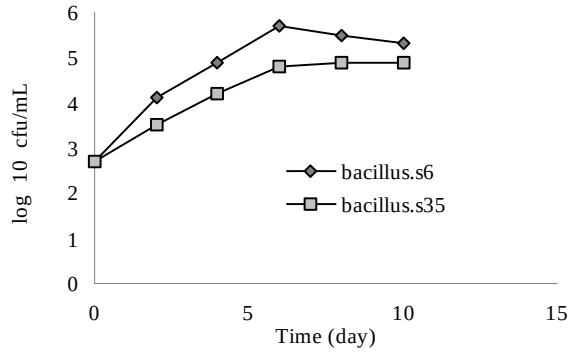

Fig. 3: Bacterial growth during degradation of crude oil (1%)

that isolated bacillus can use crude oil as the source of carbon and energy.

Emulsification test (E24)

E24 was measured for isolated *bacillus*. E24 values increased with increasing cell growth, reaching their optimum at about 32-48h and were remaining constant until the end of experiment. These results indicate that the biosurfactant biosynthesis from crude oil degrading microorganisms occurred predominantly during

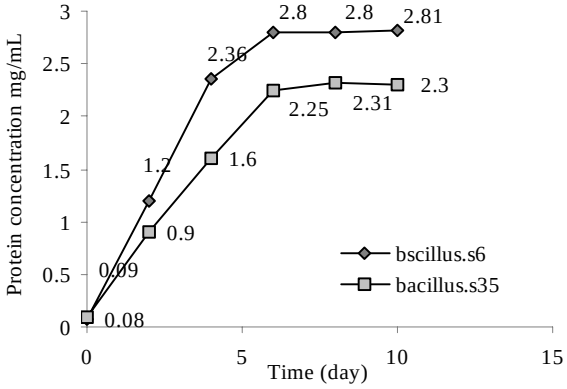

Fig. 4: Total protein estimation of *bacillus S6* and *S35* on 1% crude oil-MSM

the exponential growth phase, suggesting that the biosurfactant produced as a primary metabolite accompanying cellular biomass formation. The results show EC (Emulsification Capacity) activity of *bacillus S35* (85%) is better than *bacillus S6* (71%).

Measurement of surface tension

The microbial community caused reduction in surface tension during the experiment, indicated that these strains could produce sufficient biosurfactant. The initial surface tension was 60 (mN/m), which was reduced to 31 and 38 (mN/m) respectively by *bacillus S6* and *S35*. There was a positive correlation between the reduction of surface tension and population of microorganism. That showed biosurfactant can increase the bioavailability of crude oil and biodegradation process. The comparison of EC24 and surface tension shows that biosurfactant molecules of two isolated bacillus was different.

Enzyme activities testing of bacterial isolates

Bacterial isolates were rapid screened for enzyme productions on tested media (Table 1). Microbial extracellular enzyme activities are potentially important in the bioremediation of organically polluted sites. The results showed that enzymatic system of *bacillus S6* is more complicated than *bacillus S35* and this reflects that why *bacillus S6* shows better growth on crude oil.

Effect of degradation on pH

The initial pH of medium was 6.8 in this experiment. The pH measurement showed decrease of pH in the experimental flasks within the incubation period. This result confirmed chemical change of the

crude oil hydrocarbons which must have been changed by microbial enzymes. Microbial degradation of hydrocarbons often leads to production of organic acids and other metabolic products (bioproducts), thus the organic acids probably caused the reduction in pH.

Table 1: Production of enzymes by bacillus spp. isolated from contaminated sites

	Protease on skimmed milk	Amylase	Protease on gelatin	Urease	Lesitinase	Nitrate reductase	vp	Indole
Bacillus S6	+++	+++	+	-	-	+++	-	++
Bacillus S35	-	-	+	-	-	+++	-	-

-, negative result: no clear zone (or zone of growth); +, positive result: a clear zone (or zone of growth): All experiments were carried out in duplicate

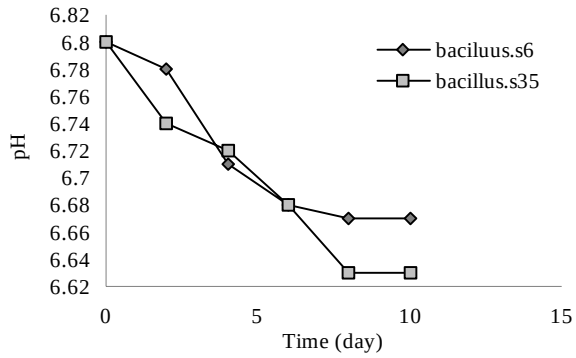

Fig. 5: pH of 1%crude oil-MSM medium for *bacillus S6* and *S35* during the experiment period

DISCUSSION

In this study, the soil samples were gathered from the crude oil contaminated sites because the capability of native bacterial population to mineralize crude oil hydrocarbons in crude oil contaminated sites was confirmed before by many scientists (Ojo, 2006; Okoh, 2003; Emtiazi, Shakarami, 2004; Kasai, 2002; Okerentugba, 2003). The growth dynamics of the organisms was determined by the optical densities, and total viable count. The results were shown in Figs. 1 to 3, and reflect the ability of isolated *bacillus* to degrade and utilize crude oil as a source of carbon and energy. This technique was used in several study to show the ability of bacteria for utilizing crude oil. (Bola, 2006; Emtiazi and Shakarami, 2004).

In the similar investigation by Rahman (2002) in England, the total viable count method used to confirm the ability of different kind of bacteria for mineralizing crude oil. The comparison of the statistics obtained in this study and other similar study shows that isolated bacillus can use crude oil and degrading that better than many kind of bacterial that isolated previously.

Another method that used in this study to demonstrate the increasing of bacterial population in culture medium was Total Protein Estimation by Bradford's method. Increasing of protein concentration during the incubation period reveals that isolated bacillus can utilize crude oil as source of carbon and energy. The initial concentration of protein observed, was 0.08±0.05 mg/mL and then increased to 2.81 mg/mL and 2.3 mg/mL for *bacillus S6* and *S35* respectively (Fig. 4). In another study, Rahman used this method to reveal the increasing of degrading crude oil bacteria in soil and confirmed the ability of these bacteria to utilize crude oil (Rahman and Street, 2002).

All isolated strains were tested for haemolytic activity, which is regarded by some authors as indicative of biosurfactant production and used as a rapid method for bacterial screening (Abu-Ruwaida 1991; Banat, 1995). After haemolysis test, stabilization of an oil and water emulsion is commonly used as a surface activity indicator. Several studies focused on high emulsifying abilities (Francy *et al*., 1991; Bicca *et al*., 1999). Highest emulsion value (water-in-oil) of about 85% was obtained from *bacillus S6*. This result is better than other similar experiments (Bodour *et al*., 2004). Identification of biosurfactant producing bacteria confirmed by measurement of surface tension.

Reduction of surface tension by isolated *bacillus* indicates that these bacillus could produce surface-active compounds . In this study the initial surface tension was 60 mN/m, which was reduced to 31 and 38 mN/m respectively by *bacillus S6* and *S35*. Similar results obtained by Banat *et al*., (1991), They isolated several bacteria which showed the ability to reduce culture-broth surface tension to values below 40 mN/m. Comparable results were obtained by Kim *et al*., 1997. Surface tension

reducing and emulsification characteristics of isolated bacillus suggest that these *bacillus* spp. are suitable for using in oil fields such as MEOR. The growth profiles showed that none of the bacterial isolates exhibited lag phases. This observation has been reported previously (Okerentugba and Ezeronye, 2003).

The enzymatic activities of isolated bacillus were investigated to identify these bacteria primarily. The obtained results shows that enzymatic system of *bacillus S6* is more complicated than *bacillus S35* and this reflects why *bacillus S6* shows better growth on crude oil. The initial pH of medium was 6.8 in this experiment. The pH measurement showed decrease of pH in the experimental flasks within the incubation period. The isolated bacteria decreased the pH of culture medium. Similar result was obtained by Matthew *et al*., 2006. It shows chemical change of the crude oil hydrocarbons and production of bioproducts and ability of isolated *bacillus* to use crude oil and generate organic acids. In the comparable study that was done by Bola, the isolated pseudomonas could decrease pH of medium from 7.5 to 6.8.

It is evident from this investigation that isolated bacillus can degrade crude oil and comparison of obtained results and existing statistics with similar studies revealed that isolated *bacillus* can use for bioremediation and elimination of crude oil pollutant from the environment. Further understanding of the metabolic process of this organism on the crude oil will increase possibilities of developing models and strategies for removing crude oil pollutants from oil-impacted environments.

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REFERENCES

Abu-Ruwaida, A. S., Banat, I. M., Haditirto, S., Salem, A., Kadri, M., (1991). Isolation of biosurfactant-producing bacteria product characterization, and evaluation. Biotechnologica., **4**: 315-324.

Balba, M. T., Al-Shayji, Y., Al-Awadhi, N., Yateem, A., (2002). Isolation and characterization of biosurfactant-producing bacteria from oil-contaminated soil., **11**: 41-55.

Banat, I. M., (1995). Biosurfactant production and possible uses in Microbial Enhanced Oil Recovery and Oil Pollution Remediation., **51**: 1-12.

Barathi, S., Vasudevan, N., (2001). Utilization of petroleum hydrocarbons by Pseudomonas fluorescens isolated from petroleum contaminated soil., **26**: 413-416.

Bicca, F. C., Fleck, L. C., Zachio, M. A., (1999). Production of biosurfactant by hydrocarbon degrading Rhodococcus rubber and Rhodococcus erythropolis., **30**: *PP.* 3.

Bodour, A. A., Gerrero, B. C., Maier, M., (2004). Structure and characterization of Flavolipids, a novel class of Biosurfactants produced by Flavolipid sp. Strain MTN11. App and Env Microbiol, **10**:1114-20.

Bradford, M. M., (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., **72**: 248-254.

Cooper, D. G., Goldenberg, B., (1999). Surface active agents from two Bacillus species .Applied and environmental microbiology., **189**: 224-229.

Del Arco, J. P., De Franca, F. P., (2001). Influence of oil contamination levels on hydrocarbon biodegradation in sandy sediment. Environ. Pollut., **1 (**10): 515-519.

Emtiazi, G., Shakarami, H., (2004). Utilization of petroleum hydrocarbons by Pseudomonas sp. and transformed Escherichia coli African J. Biotechnol., **4** (2): 172-176.

Francy, D. S., Thomas, J. M., Raymond, R. L., Ward, C. H., (1991). Emulsification of hydrocarbon by surface bacteria. J. Ind. Microbiology., **8**: 237-246.

Head, I. M., Swannell, R. P., (1999). Bioremediation of petroleum hydrocarbon contaminants in marine habitats. Curr. Opin. Biotechnol., **10**: 234 -239.

Holt, J. G., Krieg, N. R., Sneath, P., Stanley, J. T., William, S. T., (1994). Bergey's Manual of Determinative Bacteriology. Baltimore., **10**: 112-125.

Kapley, A., Heman, J., Chhatre, J. P., Shanker, R., Chakrabarti, K., (1999). Osmotolerance and hydrocarbon degradation by a genetically engineered Microbial consortium. Bioresour. Technol., **61**: 241-245.

Kim, H. S., Young, B., Lee, C., (1997). Production and properties of a lipopeptide biosurfactants., **51**: *pp*. 235.

Korda, A., Santas, P., Tenente, A., Santas, R., (1997). Petroleum hydrocarbon bioremediation. Appl. Microbiol. Biotechnol., **48**: 677-689.

Margesin, R., (2000). Potential of cold-adapted microorganisms for bioremediation of oil-polluted Alpine soils. Int. Biodeter. Biodegrad., **46**: 3-10.

Matthew, O., (2006). Hydrocarbon Degrading Potentials of Bacteria Isolated from a Nigerian Bitumen (Tarsand) Deposit. Nature and Science., **4** (3): 51-57.

Okerentugba, P. O., Ezeronye, O. U., (2003). Petroleum degrading potentials of single and mixed microbial cultures isolated from rivers and refinery effluent in Nigeria. Afr. J. Biotechnol., **2**: 288-292.

Parry, J. M., Gibson, J. R., (1988). A colour Atlas of bacillus. species., **4** (1): 17-23.

Seklemova,, E., Pavlova, A., Kovacheva., K., (2001). Biostimulation based bioremediation of diesel fuel: Field demonstration. Biodegrad., **12**: 311-316.

Urum, K., Pekdemir, T., Gopur, M., (2003). Optimum conditions for washing of crude oil-contaminated soil withbiosurfactant solutions. Process Safety and Environm.Protect.: Transact. Institut. Chem. Engin., **81**: 203-209.