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EFFECTIVENESS OF CARRIER–BASED INDIGENOUS MICROORGANISMS FOR REMEDIATION OF CONTAMINATED SOILS

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Abstract: Environmental damage due to the oil spills in the past and recent time has focused on the need for the environment friendly strategies for remediation of the contaminated site. Serial dilution pour plate method was employed for the isolation of bacteria and fungi. 1g of each soil sample was suspended in 9ml of double distilled water to make microbial suspensions (10–1 to 10–3). Dilutions of 10–1 and 10–3 were used to isolate both bacteria and fungi. Nutrient agar (NA) and potato dextrose agar (PDA) were used for the isolation of both bacteria and fungi that were used for biodegradation. Minimal salt medium was used for the biodegradation at different concentrations of crude oil (0.015, 0.0225 and 0.0375 ml). Supernatants of different concentrations at every 48 hrs were collected for spectrophometric analysis. The results were plotted into graphs using MATLAB. *Pseudomonas aeruginosa, Bacillus subtilis, Bacillus cereus* and *Pseudomonas putida* bacteria *were* isolated for the experiment. The result indicated that *Pseudomonas aeruginosa* among the bacteria isolates used performed well than other isolates as it gave the highest biodegradation value of 0.342 while *Penicillium chrysogenum* among the fungal isolates used performed well than other isolates as it gave the highest biodegradation value of 0.378. The results obtained showed that all the bacterial and fungal isolates were able to biodegrade crude oil at different concentrations and days. This study therefore shown that *Pseudomonas aeruginosa* and *Penicillium chrysogenum* are good biodegraders and are recommended for oil spillage pollution biocontrol and further molecular work is suggested to enhance their productivities.
Keywords: fungi, bacteria, biodegradation, hydrocarbon, crude oil contaminated soils

INTRODUCTION

Biodegradation generally refers to the breakdown of organic compounds by living organisms eventually resulting in the formation of carbon dioxide and water or methane. Inorganic compounds are not biodegraded, but they can be bio transformed, that is, transformed into compounds having more or less mobility or toxicity than their original form. In many cases, the biodegradation processes involve a particular microorganism that attacks a specific molecular site.

Complete and rapid biodegradation of many contaminants may require not only specific environmental conditions, but also changing conditions to satisfy the needs of the microbe. The mobility of several different metals in soil and the influence of the biodegradation process on that mobility. They have shown that active microorganisms influence the ability of soil to retain or release metals and that cysteine is an effective agent for the release of some metals from soil.

Hydrocarbon contaminants are removed from soils by bioremediation and volatilization.

In this project, bio remediation is the major technique adhered. The potential of hydrocarbon biodegradation depends on the availability of desired microorganisms. Supplementing soils with prepared cultures is practiced when the indigenous content is low.

Environmental conditions such as pH, temperature, oxygen, nutrients, and soil moisture also can influence biodegradation results. Air emissions from the "bio pile" are treated by bio filtration where the pollutants are degraded and mineralized by heterotrophic aerobic microorganisms.

Oil spills, whether on water or soil do disappear, but very little is known about what can be done to accelerate this process. The disappearance of oil from sea water could be accelerated by the addition of deficient nutrients such as nitrogen or phosphorous, or both. Suggestions have also been made for microbial seeding of spills since bacteria and fungi are the only biological species which have the metabolic capability of utilizing petroleum carbon for cell synthesis. Crude oil is essentially a mixture of carbon and hydrogen, and thus spills will result in an imbalance in the carbon–nitrogen ratio at the spill site.

For bacteria to grow efficiently, they require about 10 parts carbon to 1 part nitrogen. If the ratio is greater, e.g. 100:1 or 1,000:1, growth of the bacteria and utilization of carbon source(s) will be retarded. In addition to there being a nitrogen deficiency in oil–soaked soil, other nutrients such as phosphorus may become growth–rate limiting. Therefore, in the experiment described above, urea–phosphate, a fertilizer, was added to oil spilled on soil, thus correcting both deficiencies in one application.

Thus, oil spills were also inoculated with oil–utilizing bacteria with and without a concurrent application of the urea–phosphate amendment.

Environmental damage due to the oil spills in the past and recent time has focused on the need for the environment friendly strategies for remediation of the contaminated site.

For instance, contamination of the environment with crude oil results in pollution, in particular presents a chronic problem to commercial fisheries, recreational resources and public health. Bioremediation is suggested for remediation of contaminated soil sites because of its low cost and its ability to convert contaminants to harmless end products. Other physical and chemical processes have been used to remove spilled oil from environment; however the use of these technologies has not always been successful. Bioremediation, the use of microorganisms or microbial process to degrade environmental contaminants is among these technologies. Numerous microorganisms, including bacteria, fungi, and yeasts are known for their ability to degrade hydrocarbons. Recently, bio augmentation which involves the addition of microorganisms to enhance specific biological activity has been applied in attempts to remediate numerous environmental problems. The potential of using microorganisms for degradation of crude oil and its constituents to minimize contamination have prompted a number of researchers to study the process in laboratories. For instance, augmenting the contaminated site with appropriate bacterial inoculum is a promising technique to enhance the biodegradation of hydrocarbons.

The need for bio remediating of agricultural land have become very essential in view of the high premium placed on it as a veritable source of water, land for agriculture and good environmental/climatic condition for the people living in the community. The study was done to determine the rate of the degradability of a spilled agricultural soil which could further be used for agriculture. This was achieved through developing a conceptual design of experiment with anticipated results, isolation of bacteria and fungi using serial dilution method, purification of bacteria and fungi, characterization of bacteria isolates and determination of the rate of bio degradability potential of both bacteria and fungi on a separate graph using matrix laboratory (MATLAB).

Crude oil is a naturally occurring flammable liquid consisting of a complex mixture of hydrocarbon of various molecular weights and other liquid organic compounds, which are found in geologic formations beneath the Earth's surface (Guerriero et al., 2011). The use of fossil fuels such as crude oil can have a negative impact on Earth's biosphere, releasing pollutants and greenhouse gases into the air and damaging ecosystems through events such as oil spills. Concern over the depletion of the earth's finite reserves of oil, and the effect this would have on a society dependent on it, is a field known as peak oil (WHO, 2004).

Generally any oil spill is most harmful and detrimental to the surrounding environment, but the crude oil toxins that escape into the atmosphere pose a possible health risk for individuals living in and around the affected area. Health risks are most prominent in the very young, the elderly and those who suffer from asthma. After an oil spill, however, contamination is more concentrated and widespread. Oil remediation workers are at a particularly high risk of sickness due to their continued and direct exposure to petrochemicals. In addition, the toxins quickly disperse into the air and are inhaled by individuals living in coastal cities and, if the wind is right, those living several miles inland as well (WHO, 2004).

— Components of Crude Oil

Crude oil is essentially a mixture of many different hydrocarbons, all of varying lengths and complexities. In order to separate the individual components that make up the raw natural resource, the crude oil must be fractionally distillated so that chemical components can be removed one at a time according the their boiling points (Matveichuk, 2004).

— Composition of Crude Oil

In its strictest sense, petroleum includes only crude oil, but in common usage it includes all liquid, gaseous, and solid such as paraffins hydrocarbons. Under surface pressure and temperature conditions, lighter hydrocarbons methane, ethane, propane and butane occur as gases, while pentane and heavier ones are in the form of liquids or solids. However, in an underground oil reservoir the proportions of gas, liquid, and solid depend on subsurface conditions and on the phase diagram of the petroleum mixture (Itah and Essien, 2005).

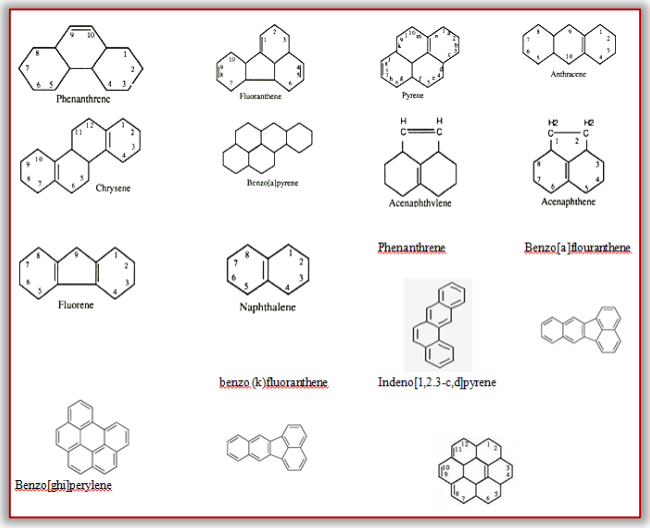

Figure 1: Structure of Health Implicated Polyaromatic Hydrocarbon

The proportion of light hydrocarbons in the petroleum mixture varies greatly among different oil fields, ranging from as much as 97 per cent by weight in the lighter oils to as little as 50 per cent in the heavier oils and bitumen (Leonardo, 2005).

The hydrocarbons in crude oil are mostly alkanes, cycloalkanes and various aromatic hydrocarbons while the other organic compounds contain nitrogen, oxygen and sulphur, and trace amounts of metals such as iron, nickel, copper and vanadium (Aldis and Anne, 2004). There are different types of hydrocarbon present in the composition of crude oil, which are Naphthalene, Acenaphthylene, Acenaphthene, Flourene, Anthracene, Phenanthrene, Fluoranthene, Pyrene, Benzo(a) anthracene, Chyrsene,

Benzo(a) flouranthene, Benzo(k) flouranthene, Benzo(a) pyrene, Dibenzo(ah)perylene, Benzo(ghi)perylene, Indeno (1,2,3–cd)pyrene. (Encyclopedia of Industrial Chemistry, 2002), proportions of chemical element vary over fairly narrow limits as shown in Table 1.

Table 1: Composition of elements present in crude oil by weight Composition by weight

Element	Percent range
Carbon	83 to 87%
Hydrogen	10 to 14%
Nitrogen	0.1 to 2%
Oxygen	0.05 to 1.5%
Sulfur	0.05 to 6.0%
Metals	< 0.1%

Table 2: Composition by weight of hydrocarbons present in crude oil

Hydrocarbon	Average	Range
Paraffins	30%	15 to 60%
Naphthenes	49%	30 to 60%
Aromatics	15%	3 to 30%
Asphaltene	6%	Remainder

The oil sands resources are called unconventional oil to distinguish them from oil which can be extracted using traditional oil well methods. Between them, Canada and Venezuela contain an estimated 3.6 trillion barrels (570×109 m3) of bitumen and extra–heavy oil, about twice the volume of the world's reserves of conventional oil (OPEC, 2006).

The heavier crude oils have too much carbon and not enough hydrogen; these processes generally involve removing carbon from or adding hydrogen to the molecules, and using fluid catalytic cracking to convert the longer, more complex molecules in the oil to the shorter, simpler ones in the fuels (Marcel, 1999).

— Air Pollution

About ninety–five percent of waste gases from the production fields and operation are flared. Gas flaring pollutes the air and it is common practice among companies in Nigeria especially in the Niger–Delta region which is hazardous to the ozone layer of the area and leading to climate change (IPCC, 2007). This is the major source of air pollution in the area as well untreated waste disposal on the environment.

According to Uyigue and Agho (2007), there are about 123 flaring sites in the region making Nigeria one the highest emitter of greenhouse gases in Africa and releasing some 45.8 billion kilowatts of heat are discharged into the atmosphere of the Niger–Delta from 1.8 billion cubic feet of gas everyday (Aaron, 2006). This is environmentally unethical and has contributed significantly to the degradation of the environment in the region.

This practice have also altered the vegetation of the area, replacing natural vegetation with stubborn grasses and the presence of these grasses indicates that the soil is no longer fertile for cultivation of crops. A major example could be seen in Opuama and Sekewu communities in the Warri North Local Government Area of Delta State in the region. It is evident that gas flaring has affected the ozone layer of the region leading to climate change that is unhealthy to crops cultivation (IPCC, 2007).

— Major Air Pollutants

Air pollution is a real public health and environmental problem that can lead to other things like global warming, acid rain, and the deterioration of the ozone layer. Table 3 stated some common pollutants, their sources, and their effect on the environment (Levy, 2007).

— Effect of crude oil in Nigeria

The Niger Delta is one of the 10 most important wetland and coastal marine ecosystems in the world and is home to some 31 million people (NDTC, 2008). The Niger Delta is also the location of massive oil deposits, which have been extracted for decades by the government of Nigeria and by multinational oil companies. Oil has generated an estimated $600 billion since the 1960s (Wurthmanm, 2006). Despite this, the majority of the Niger Delta's population lives in poverty. The United Nations Development Programme (UNDP) describes the region as suffering from "administrative neglect, crumbling social infrastructure and services, high unemployment, social deprivation, abject poverty, filth and squalor, and endemic conflict."

The majority of the people of the Niger Delta do not have adequate access to clean water or health–care (UNDP, 2006). Their poverty, in contrast with the wealth generated by oil, has become one of the world's starkest and most disturbing examples of the "resource curse" (UNDP, 2006). For the people of the Niger Delta, environmental quality and sustainability are fundamental to their overall wellbeing and development. According to UNDP, more than 60 per cent of the people in the region depend on the natural environment for their livelihood. For many, the environmental resource base, which they use for agriculture, fishing and the collection of forest products, is their principal or sole source of food (UNDP, 2006). Pollution and environmental damage, therefore, pose significant risks to human rights.

Oil spills, waste dumping and gas flaring are endemic in the Niger Delta. This pollution, which has affected the area for decades, has damaged the soil, water and air quality. Hundreds of thousands of people are affected, particularly the poorest and those who rely on traditional livelihoods such as fishing and agriculture. The human rights implications are serious, under–reported and have received little attention from the government of Nigeria or the oil companies. According to a study carried out by a team of Nigerian and international environmental experts in 2006, (NCF, 2006) the Niger Delta is "one of the world's most severely petroleum–impacted ecosystems". They stated: "The damage from oil operations is chronic and cumulative, and has acted synergistically with other sources of environmental stress to result in a severely impaired coastal ecosystem and compromised the livelihoods and health of the region's impoverished residents."

The Niger Delta has suffered for decades from oil spills, which occur both on land and offshore. Oil spills on land destroy crops and damage the quality and productivity of soil that communities use for farming. Oil in water damages fisheries and contaminates water that people use for drinking and other domestic purposes (NDHD, 2006).There are a number of reasons why oil spills happen so frequently in the Niger Delta. Spills result from corrosion of oil pipes, poor maintenance of infrastructure, spills or leaks during processing at refineries (WB Report, 1995), human error and as a consequence of deliberate vandalism or theft of oil (Richard, 2008).In August and December 2008, two major oil spills disrupted the lives of the 69,000 or so people living in Bodo, a town in Ogoni land in the Niger Delta. Both spills continued for weeks before they were stopped. Three years on, the prolonged failure of the Shell Petroleum Development Company of Nigeria, a subsidiary of Royal Dutch Shell, to clean up the oil that was spilled, continues to have catastrophic consequences for the Bodo community. The lives of tens of thousands of people have been directly affected by the spills and the ongoing pollution. Many are worried about their health and are afraid to eat locally caught fish or drink water from streams or rain water, as they did before the oil spills.

— Crude Oil's Toxins

Any oil spill is detrimental to the surrounding environment, but the crude oil toxins that escape into the atmosphere pose a possible health risk for individuals living in and around the affected area (WHO, 2003). Crude oil is a complex mixture of chemical constituents including various alkanes (butane, pentane, and hexane); aromatic hydrocarbons (benzene, ethyl benzene, toluene, and xylenes); cycloalkanes; other nitrogen, oxygen, and sulfur compounds (hydrogen sulfide); and trace metals such as iron, nickel, copper and vanadium (LDHH, 2010). Some constituents of crude oil can have significant toxicity. For example, several aromatic hydrocarbons are considered to be human carcinogens (Joseph, 2004).

The International Agency for Research on Cancer (IARC) indicates that for crude oil, there is inadequate evidence for the carcinogenicity in humans, although there is limited evidence for carcinogenicity in experimental animals (Matt, 2005). Hydrocarbon exposures from crude oil constituents will vary based on its exposure to the atmosphere, time in the marine aquatic and coastal environment, treatments with dispersants and interaction of the chemicals, wave action and heat. Generally, the more "aged" or "weathered" crude oil is (by mixing with seawater and traveling long distances from the source), the lower are the concentrations of volatile organic compounds (VOCs) (NIOSH, 2005). Although it generates less VOCs, weathered crude oil still contains harmful chemicals which can cause skin irritation and other irritant reactions. Thus, use of gloves and protective clothing is recommended to minimize skin contact with weathered oil, including oil deposited on the shore (tarballs) (WHO, 2003). Appropriate hand hygiene facilities should be readily available to clean incidental skin exposures (NIOSH, 2005). Weathered crude oil is unlikely to pose an inhalation risk although a potential risk does exist for it to be aerosolized into respirable airborne droplets or volatilized by activities such as pressure washing. Even though detection of hydrocarbon "odors" is common in areas contaminated by crude oil, odor is not a reliable indication of a health hazard. Some individuals, though, are bothered by odors and can develop symptoms (e.g., may report dizziness, nose and throat irritation, headache and/or nausea) (WHO, 2003). These individuals may need medical evaluation when symptoms occur, especially if severe or persistent. Individuals with severe or persistent symptoms should be relocated to perform tasks where symptoms can be alleviated (NIOSH, 2005).

Studies of tanker oil spill responses have reported adverse health effects in response workers. These studies may underestimate the health effects associated with the Deepwater Horizon Response activities since the magnitude and duration of the Response is unprecedented. In addition, there is an incomplete understanding about the human health toxicity associated with the use of large amounts of dispersant, about the toxicity of the mixed exposure to large amounts of crude oil, dispersants and combustion products together and the cumulative effect of such exposures occurring over a long duration (Mendez, 2010). Since knowledge about potential inhalational exposures to the mixed exposure of crude oil, dispersant and combustion products associated with the Deepwater Horizon Response work is incomplete and still evolving, NIOSH and OSHA believed it was prudent to reduce the potential for adverse health effects by the responsible use of engineering controls, administrative controls and PPE, including respirators when appropriate (NIOSH, 2005). In the absence of comprehensive and coordinated health surveillance among workers and volunteers, and the absence of interpretable, quantitative exposure data, NIOSH and OSHA recommended that employers take precautions sufficient to ensure workers are protected from the chemical, physical and psychological hazards posed by the Deepwater Horizon Response (NIOSH and OSHA, 2005).

Environmental damage due to the oil spills in the past and recent time has focused on the need for the environment friendly strategies for remediation of the contaminated site. For instance, contamination of the environment with crude oil results in pollution, in particular presents a chronic problem to commercial fisheries, recreational resources and public health. Bioremediation is suggested for remediation of contaminated soil sites because of its low cost and its ability to convert contaminants to harmless end products (Rahman et al., 2002; Sathishkumar et al., 2008). Other physical and chemical processes have been used to remove spilled oil from environment; however the use of these technologies has not always been successful (Aldrett et al., 1997). Bioremediation, the use of microorganisms or microbial

process to degrade environmental contaminants is among these technologies (Boopathy, 2000). Numerous microorganisms, including bacteria, fungi, and yeasts are known for their ability to degrade hydrocarbons (Swannell and Head, 1994). Recently, bio augmentation which involves the addition of microorganisms to enhance specific biological activity has been applied in attempts to remediate numerous environmental problems (Vogel, 1996). The potential of using microorganisms for degradation of crude oil and its constituents to minimize contamination have prompted a number of researchers to study the process in laboratories. For instance, augmenting the contaminated site with appropriate bacterial inoculum is a promising technique to enhance the biodegradation of hydrocarbons.

Although pesticides are hydrocarbon pollutants of the soils, the main sources of hydrocarbon pollution are the spills and leaks of petroleum products (Potter, 1993). The Exxon Valdez oil spill in South Central Alaska is an example (Pritchard et al., 1992). In Nigeria, the exploration and exploitation practices and the breaking of oil pipes lead to incessant pollution especially in the Niger Delta area and Southern part of Nigeria (Salu, 1999). These spills have the largest immediate and economic impact as they harm, to a large extent, the ecosystem more than just the isolated location. In many spills involving tankers or offshore oil wells, some of the spills catch fire and consequently their combusting results in emission of large quantities of toxic ash which is detrimental to human health.

In recent times, the number of microbiological research has been devoted to bioremediation of oil–contaminated sites using various microbial species (Atlas, 1981). Notable among them were the bacterial species of Arthrobacter (Edgehill and Finn, 1982), Flavobacterium (Saber and Crawford, 1985), Sphingomonas (a novel Pseudomonas sp) (Radehaus and Schimidt, 1992), Pseudomonas spp. (Leung et al., 1997) and Acinetobacter (George–Okafor et al., 2005). Fungal species such as Trichoderma (Cserjesi and Johnson, 1972) and Phanerochaete (Andrea et al., 2001) have been implicated in hydrocarbon biodegradation.

Fungal bioremediation has been successful for clean–up of pentachlorophenol (PCP), a wood preservative and polycyclic aromatic hydrocarbon (Andrea et al.2001). The advantages associated with fungal bioremediation lay primarily in the versatility of the technology and its cost efficiency compared to other remediation technologies (such as incineration, thermal desorption, extraction) (Aust, 1990).

The use of fungi is expected to be relatively economical as they can be grown on a number of inexpensive agricultural or forest wastes such as corncobs and sawdust. More so, their utilization is a gentle non–aggressive approach. The application of bioremediation capabilities of indigenous organisms to clean up pollutants is viable and has economic values (Bijofp, 2003).

MATERIALS AND METHODS

— Materials and Equipments

The materials and equipments used for isolation of bacterial were; Disposable petri dishes, beaker, inoculation loop, Conical flask, measuring cylinder, slant bottles, gin bottles, cotton wool, funnel, foil paper, sieve, test–tube, distilled water, spatula, weighing balances, autoclave, shaker, centrifuge, spectrophotometer, ethanol, paper tape, NA (nutrient agar), (PDA) Potato Dextrose Agar and Incubator.

— Collection of Soil Samples

Soil sample was collected from local mechanic garage along isale general, Ogbomoso, Oyo–State. The soil sample was taken to Ladoke Akintola University of Technology central Laboratory for analysis.

— Preparation of nutrient Agar and Potato Dextrose Agar

Potato Dextrose Agar (PDA) and Nutrient Agar (NA) used in this study were prepared according to the manufacturer's specification i.e. 39 g of PDA and 28 g of NA in 1000 ml of distilled water each. Bacterial contamination was inhibited by aseptically adding 2g of tetracycline to 1000 ml of the sterile medium prior to pouring into sterile petri dishes, while fungi contamination inhibition was done by aseptically adding 2 ml of nystatin to 1000 ml of the sterile medium prior to pouring into sterile petri dishes for bacteria plates. The media were prepared, mixed thoroughly and sterilized by autoclaving at 121^{0}C for 15 minutes.

Figure 2: Solidified Potatoes Dextrose Agar

Figure 3: Solidified Nutrient Agar

— Isolation of Bacteria and Fungi

Serial dilution pour plate method was employed for the isolation of bacteria and fungi.1 g of each soil sample was suspended in 9ml of double distilled water to make microbial suspensions (10–1 to 10–3). Dilutions of 10–1 and 10–3 were

used to isolate both bacteria and fungi. 1ml of microbial suspension of each concentration was added to sterile Petri dishes containing 15ml of sterile Potato Dextrose Agar for fungi isolation and Nutrient Agar for bacteria isolation (Waksman, 1992). After the plates have been solidified, the petri dishes for fungi were incubated at 30^{0}C for 4 days while bacteria petri dishes were incubated 37^{0}C for 24 hours. After the organisms have grown, both bacterial and fungal colonies were pure cultured.

— Purification of Bacteria and Fungi

Re–streaking on fresh plate containing solidified NA and PDA was done to obtain pure isolates. This sub culturing was continued until pure bacterial and fungal isolates were obtained. After pure isolates have been obtained, they were stored in different slant bottles containing solidified NA and PDA respectively. The storage was done based on their colonial morphology on NA and PDA plates before 24 hours. After pure isolates have been obtained, they were stored in slant bottles for characterization.

— Screening for the Biodegradability of both Bacterial and Fungal Isolates

After the plates have solidified, pure isolates of each organism (i.e. bacteria and fungi) was inoculated into the solidified plates. Bacterial plates were incubated at 37^{O}C for 48 hours while fungal plates were incubated at 30^{0}C for 6 days. At 48 hours of incubating bacteria, bacteria growth was observed on the plates while no growth was observed on fungal plates after 6 days of incubation. Therefore, bacterial isolates were stored for characterization and biodegradation of Crude Oil at different concentrations.

Figure 4: Bacteria plates incubated at 37^{0}C for 48 hour

Figure 5: Fungi plates incubated at 30^{o}C for 72 hours

— Inoculum Development

A loopful of the inoculum was taken from the NA slant bottles and inoculated into a sterilized Nutrient broth inside gin bottles. All the gin bottles were incubated in the incubator at 37^{0}C for 24 hours.

— Biodegradation Crude Oil and Minimal Salt Medium

Chemically defined minimal salt medium was prepared by weighing different five salts and the crude oil at various concentrations (i.e. 5 ppm, 15 ppm and 25 ppm) into 1500 ml of distilled water in three (3) bowls.

Table 3: The constituents of Crude Oil selective medium

SALT	CONCENTRATION (g/l)
$MgSO_4$	2
$NaNO_3$	2
NH_4Cl	2
KH_2PO_4	2
$CaCO_3$	2
Crude Oil	0.015, 0.0225, 0.0375

For the substrate, crude oil concentration was varied as 0.015g, 0.0225 and 0.0375g. After the media had been prepared, 90ml of the each medium was measured into gin bottles each and were sterilized at 121oC for 15 minutes and allowed to be properly cooled.

— Inoculation of Inoculum into Minimal Medium

After sterilization and the bottles had been properly cooled, 10ml of the bacteria isolates was inoculated into the minimal salt medium prepared into the bottles. The bottles were then transferred into the shakers in order to evenly shake or distribute the medium with the inoculum. The bottles were then later stored inside an incubator of 370 C. Supernatant for each of the isolate was harvested at every 48 hours for a period of 4 days.

Figure 6: Inoculation of bacteria into minimal salt medium

Figure 7: Inoculation of fungi into minimal salt medium

— Extraction of Bacterial from Minimal Salt Medium

After the incubation, pH of the minimal salt medium at different hour was determined also, the minimal salt medium at 2 days and 4 days were harvested by the techniques.

— Centrifugation

At 2 days and 4 days after inoculation, the supernatant were collected by centrifugation at 500rpm for 10minutes. The clear supernatant was stored in universal bottle and kept in refrigerator, after which it was subjected to estimate for spectrophotometry analysis.

— Spectrophotometry analysis for the Supernatants Collected

The clear supernatant of the three (3) concentrations collected from the formation media were placed into the universal bottles and spectrophotometric analysis was done with the absorbance read taken as 540 nm.

— Characterization of Bacterial Isolates

Colonial characteristics of the bacterial isolates were determined using parameters such as size, elevation, pigment, surface, opacity, edge and shape. Cellular characteristics of the isolates were determined through the following experiments: Gram's staining, Motility test, Spore staining, Capsule staining, Catalase test, Oxidase test and Methyl red test. Others include Indole test, Starch hydrolysis test, Citrate utilization test, Sugar fermentation test and Oxygen relationship test.

RESULTS AND DISCUSSION

Bacterial strains were isolated from soil samples collected from the site. The pure cultures of all the isolated bacterial strains were used for the biodegradation. The following are the bacteria isolated; Pseudomonas aeruginosa, Bacillus subtilis, Bacillus cereus and Pseudomonas putida. The ability of all the bacterial isolates to degrade the crude oil at different concentrations (0.015 ml/L, 0.0225 ml/L and 0.0375 ml/L) was analyzed spectrophotometrically.

Figures 8 to 15 showed the results obtained from the experiment at every 48 hours for a period of 4 days. All the bacterial and fungal strains moderately degraded the crude oil at different concentrations. Crude oil in the medium was reduced as a result of the biodegradation potential by the various bacterial and fungal isolates compared with the control that has no organism. Biodegradability of the crude oil by the bacterial and fungal isolates irrespective of the concentrations was noted, although some organisms could not biodegrade crude oil at higher concentrations. At the second and fourth day of incubation, the highest rate of biodegradation of crude oil by the bacteria isolate Bacillus subtilis gave 0.27 and 0.341 respectively at 0.0375 mg/ml; this indicates that biodegradation increases as the incubation periods and concentration increase as shown in Figure 8. Also at the second and fourth day of incubation, the highest rate of biodegradation of crude oil by the bacteria isolate Bacillus cereus gave 0.228 and 0.165 respectively at and 0.015 mg/ml and 0.0375 mg/ml respectively but it gave the least of 0.096 and 0.165 at 0.0225 mg/ml and 0.0375 mg/ml respectively, this indicates that biodegradation increases as the incubation periods increases but decreases as concentration increases as shown in Figure 9.

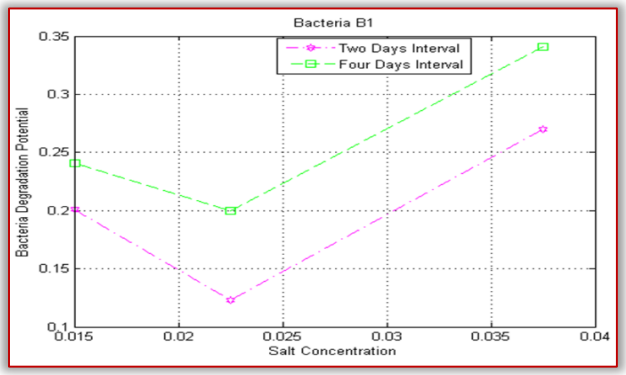

Figure 8: Biodegradation of Crude Oil by Bacillus subtilis at Different concentrations

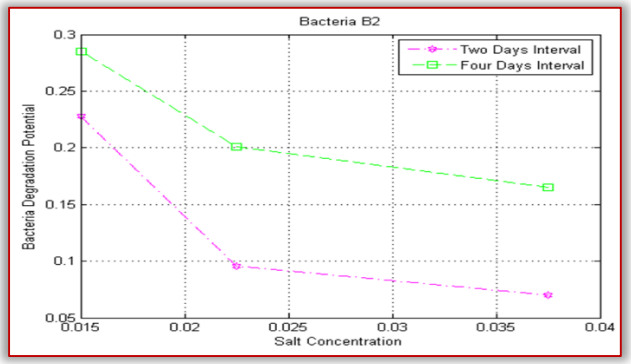

Figure 9: Biodegradation of Crude Oil by Bacillus cereus at Different concentrations

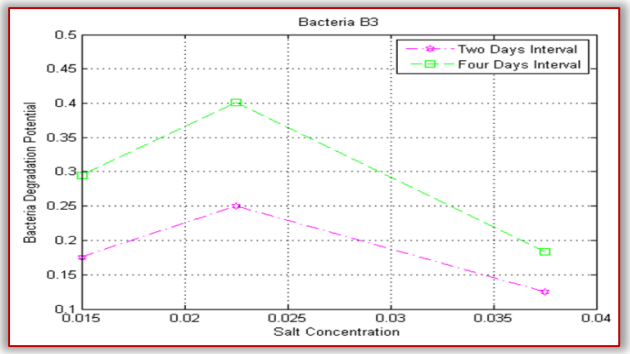

Figure 10: Biodegradation of Crude Oil by Pseudomonas putida at Different concentrations

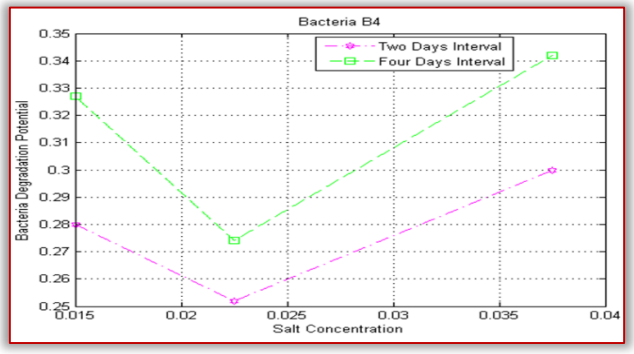

Figure 11: Biodegradation of Crude Oil by Pseudomonas aeruginosa at Different concentrations

In Pseudomonas putida, at the second and fourth day of incubation, the highest rate of biodegradation of crude oil by the bacteria isolate gave 0.176 and 0.165 respectively at 0.015 mg/ml and 0.0375 mg/ml respectively but it gave the least of 0.125 and 0.184 at 0.0375 mg/ml respectively, this indicates that biodegradation increases as the incubation periods increases but decreases as concentration increases as shown in Figure 10. At the second and fourth day of incubation, the highest rate of biodegradation of crude oil by the bacteria isolate Pseudomonas aeruginosa gave 0.300 and 0.342 respectively at 0.0375 mg/ml; this indicates that biodegradation increases as the incubation periods and concentration increase Figure 11.

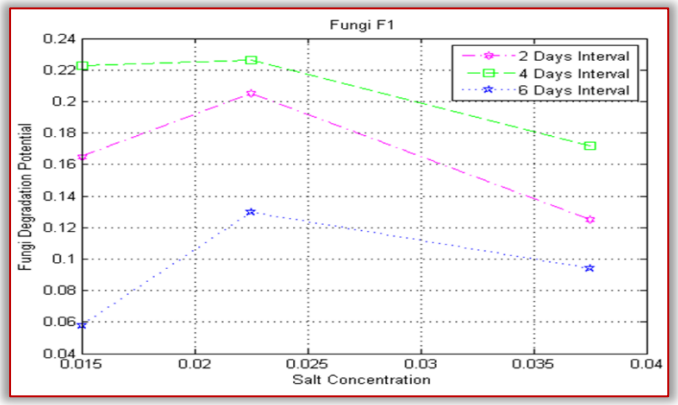

Figure 12: Biodegradation of Crude Oil by Aspergillus niger at Different concentrations

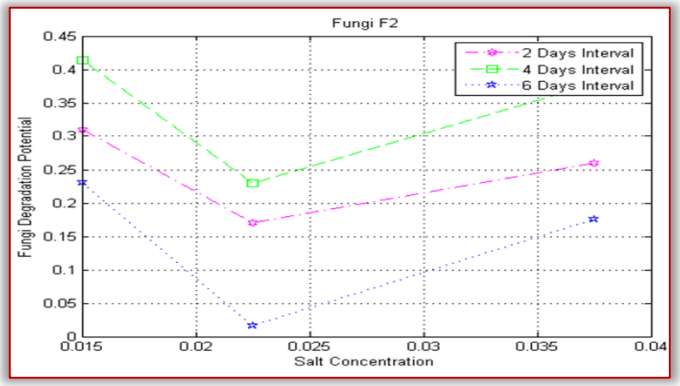

Figure 13: Biodegradation of Crude Oil by Aspergillus flavus at Different concentrations

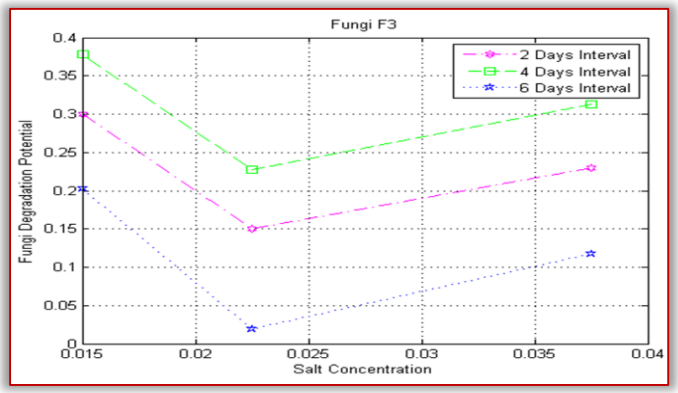

Figure 14: Biodegradation of Crude Oil by penicillium chrysogenum at Different concentrations

In the case of fungal isolates, at the second and fourth day of incubation, the highest rate of biodegradation of crude oil by the fungal isolate Aspergillus flavus gave 0.205 and 0.226 respectively at 0.0225 mg/ml but it gave least of 0.125 and 0.172 at 0.0375 mg/ml respectively, while on the sixth day the results at all concentrations reduced. This indicates that biodegradation decreases as the incubation periods and concentration increase especially after the fourth day of incubation.

At the second and fourth day of incubation, the highest rate of biodegradation of crude oil by the fungal isolate Aspergillums Niger gave 0.310 and 0.414 respectively at 0.015 mg/ml but it gave least of 0.170 and 0.229 at 0.0225 mg/ml respectively, while on the sixth day the results at all concentrations reduced. This indicates that biodegradation decreases as the incubation periods and concentration increase especially after the fourth day of incubation. Also, at the second and fourth day of incubation, the highest rate of biodegradation of crude oil by the fungal isolate Peniccilum chrysogenum gave 0.300 and 0.378 respectively at 0.015 mg/ml but it gave least of 0.195 and 0.228 at 0.0375 and 0.228 mg/ml respectively, while on the sixth day the results at all concentrations reduced. This indicates that biodegradation decreases as the incubation periods and concentration increase especially after the fourth day of incubation.

The degradation of crude oil by bacterial and fungal strains isolated from oil contaminated soil; the microorganisms implicated in oil degradation are widely distributed in nature and have been isolated from soil and water ecosystems with their oil degrading potentials (Bello, 2007).

The microorganisms capable of utilizing oil and oil products as a sole source of carbon and energy occur practically everywhere in air, water and soil (Oliver and Magot, 2005). It is estimated that in 1 g of unpolluted soil, there are only 100 to 1,000 cells of hydrocarbon degrading microorganisms, whereas, in 1 g of soil polluted by oil, their number increases to 1×106 to 5×07 cells, especially if pollution occurred repeatedly and during a long time (Rosenberg and Ron, 1996). Taxonomic characteristics of these isolates identified them as Bacillus spp and Pseudomonas spp.

CONCLUSIONS

Bioremediation is a cheap and easy method to reduce oily sludge contamination while the use of microbial inoculants is a common practice, which enhances the rate of biodegradation. The study demonstrated that, if suitably developed, application of a carrier–based indigenous microorganism like Pseudomonas spp and Bacillus spp can be used to remediate soil contaminated with crude oil. Maintenance of proper soil conditions is an essential aspect to be looked into and needs to be studied in further detail when taking up such studies because soil conditions influence the survival of the microorganisms. Bacteria and Fungi show tremendous diversity and adaptability in utilization of different organic molecule as a carbon source; however their abilities to degrade a specific hydrocarbon as a

source of energy and or biomass may differ. The chemical composition of a crude oil may also be a factor in determining the type of bacteria and fungi, which may grow on it.

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