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Assessment of percentage bioremediation of Petroleum Hydrocarbon contaminated soil with biostimulating agents

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Abstract

Bioremediation of petroleum hydrocarbon using environmentally friendly petroleum hydrocarbon impacted media option has ever since its discovery continued to elicit research interest in environmental management and pollution control. The research aimed at identifying the comparatively suitable biostimulating agent that is environmental friendly in bioremediation of petroleum hydrocarbon. One of such option is the use of goat feces and fertilizer as a biostimulating agent. A combination of treatments consisting of the application of Goat feces, and chemical fertilizer (NPK) was evaluated during a period of 10 days of remediation. Each contained petroleum hydrocarbon or crude oil mixture in the soil as a sole source of carbon and energy. After ten days of remediation, the bioremediation potential of the different treatment samples on the 5th day were; uncontaminated soil (1.25mg/l) < Contaminated soil + Goat faces + Fertilizer (2.60mg/l) < contaminated soil (3.20mg/l) < uncontaminated soil + crude oil (3.30mg/l) < uncontaminated soil + crude oil + Fertilizer (5.43mg/l) < Contaminated soil + Goat faces (6.40mg/l) < uncontaminated soil + crude oil + Goat faces + Fertilizer (7.80mg/l). Evaluatig the actual amount of petroleum hydrocarbon degraded during the study, it shows that out of the seven experimental set-up at day 15, contaminated soil + goat feces have the highest (8.27mg/l) while uncontaminated soil + crude oil was lowest (3.80mg/l) table 2. The percentage (%) bioremediation reveals; Contaminated soil + goat feces (21.01%) > Uncontaminated soil +crude oil + fertilizer + goat feces (20.78%) > Contaminated soil (15.16%) > Uncontaminated soil +crude oil + fertilizer (12.95%) > Uncontaminated soil (12.88) > Uncontaminated soil +crude oil (9.65%) > Contaminated soil +crude oil + fertilizer + goat feces (7.52%). *Pseudomonas* sp. and *Bacillus* sp. having highest occurrence followed by *Arthrobacter* sp., fungal isolates were; *Penicillium* sp., *Aspergillus* sp., *Mucor* sp. *and Rhizopus* sp. This present work showed greater effectiveness goat feces than NPK fertilizer, high moisture content in the *fresh* goat feces extends the nutrient depletion time of organic biostimulating agents thus enhancing bioremediation. Conclusively, results revealed that biostimulating agent - goat feces have the highest bioremediation potential and is more environmental friendly. It is therefore recommended that companies and refineries whose activities contaminate soil with petroleum products should be encouraged to simply seed contaminated sites with *fresh* goat feces.

Keywords: Petroleum hydrocarbon, bioremediation, biostimulating agent, NPK fertilizer, goat feces

Introduction

Bioremediation is the biological treatment systems to destroy, or reduce the concentration of hazardous waste from contaminated sites. The increase use of petroleum has generated various sources of pollution in soil, air and water, causing several problems for the environment (Vidali, 2001). Oil pollution is referred to as a partial or complete destruction of the aesthetic quality of the environment by introduction of crude oil. The increase in demand for crude oil as a source of energy as a primary raw material for industries has resulted in an increase in its production, transportation and refining which in turn has resulted in gross pollution of environment (Nano *et al*., 2003).

Bioremediation is considered a non-destructive, cost-and treatment-effective and sometimes logistically favourable cleanup technology, which attempts to accelerate the naturally occurring biodegradation of contaminants through the optimization of limiting conditions. Bioremediation is an option that offers the possibility to destroy or render harmless various contaminant using natural biological activity. As such, it uses relatively low-cost, low-technology techniques, which generally have a high public acceptance and can often be carried out on site (Alexander, 1995).

Bioremediation of petroleum hydrocarbon contaminated soil relies on the petroleum degradation ability of the microbial consortium resident in the soil, however; the choice of fossil fuel materials used in energy production, transportation etc is directly responsible for the increase in carbondioxide and other gases resulting in the current trend of global warming (Yerushalmi *et al*., 2003).

The application of biotechnological processes involving microorganisms, with the objective of solving environmental pollution problems, is rapidly growing, in recent decades, where petroleum and its by-products are concerned. Bioremediation process which takes advantages of microbial degradation of organic and inorganic substances, presents countless advantages in relation to other processes employed to remove pollution such as extraction with solvents addition of chemical oxidizers, etc. (Demnerova *et al*., 1995; Van Gestel *et al*., 2001; Gogoi *et al*., 2003; Nano *et al*., 2003; Morelli *et al*., 2005).

One approach to restoring contaminated soil is to make use of microorganisms that is able to degrade the toxic compounds in a bioremediation process. However, it is known that hydrocarbon biodegradation in soil can be limited by many factors, such as nutrients, pH, temperature, moisture, oxygen, soil properties (Bardi *et al*., 2000; Semple *et al*., 2001; Sabate *et al*., 2004; Ghazali *et al*., 2004; Walter *et al*., 2005 Atlas and Bertha 2006).

Recommendations have been advocated for the microbial seeding of oil spills, because bacteria and fungi are the only biological species which have the metabolic capability of utilizing petroleum carbon. Also, oil spills result in an imbalance in the carbon-nitrogen ratio at the spill site, because crude oil is essentially a mixture of carbon and hydrogen. This causes a nitrogen deficiency in an oil-soaked soil, the utilization of carbon source(s). (Walter *et al*., 2005). Nutrients such as nitrogen, phosphorus, and iron play a much more critical role than oxygen in limiting the rate of biodegrading in soil. Nutrients amendment in a high close can accelerate the initial oil degradation rate, and this may shorten the treatment period to clean up to the

contaminated environments (Oh *et al*., 2001). Previous studies suggest nutrient supplementation stimulates bioremediation by increasing microbial biomass (Sanchez *et al*., 2000; Duncan *et al*., 2003, Maki *et. al*., 2003; Sarkec *et.al*., 2005).

The effects of nutrients (NPK fertilizer and goat manure), and biostimulation of indigenous soil microorganisms on the bioremediation of contaminated soil have been investigated (Vasudevan and Rajaram, 2001; Gogoi *et al*., 2003; Coulon *et al*., 2005; Ayotamuno *et al*., 2006; Sang-Hwan *et al*., 2007).

Crude oil pollution tends to persist in soils until remediation measures, involving the application of nutrients, are restored to, because oxygen and nitrogen are limited factors in all types of petroleum degradation. The application of a fertilizer and goat manure were as effective as the use of bioaugmentation with indigenous hydrocarbon utilizing bacteria. However, a combination of treatments, consisting of the application of fertilizers, goat manure on bioremediation of a crude oil contaminated soil and on petroleum hydrocarbon contaminated soil was evaluated by Ayotamono *et al*., (2006). This study was designed to evaluate percentage bioremediation of Petroleum Hydrocarbon contaminated soil with bio-stimulating agents.

Material and Methods

Source of Samples – Source of soil sample:

The soil materials selected for this study were from different sites from Rivers State University of Science and Technology Port Harcourt. Contaminated soil sample were collected from a generator premises used for energy supply over several years, while uncontaminated soil sample were collected from an undeveloped fallow area (to serve as *control*) about 250meters from the site A. The generator house uses hydrocarbon-containing materials extensively including diesel, and other hydrocarbon products. Soil samples were collected from multiple sites within an area and mixed to produce composite samples. The soil were collected from the surface to a depth of 3cm with a sterile spatulas, using sterilized black polythene bags and it was transported to the laboratory. Processing of the soil samples began immediately upon it arrival at the laboratory. They were sieved through a (<2mnn) filter and air dried, the moisture content were analyzed after sieving.

Source of Crude Oil

The crude oil used is a Bonny light crude oil, it is black in colour and was obtained from an oil company in Bonny, Rivers State and it was collected with a large sterile plastic container.

Source of Nutrients

Goat faces were aseptically collected from the slaughter and fertilizers (NPK) were bought from Mile 3 market, situated in Diobu, Port Harcourt, Rivers State.

Soil preparation and application of crude oil and nutrients

Soil were collected in two places, one batch is a hydrocarbon-contaminated soil while the other batch is uncontaminated soil. 2500g of the contaminated soil were weighed into 3 batches while 2500g of the uncontaminated soil were weighed into 4 batches. However, different treatments were considered for each soil batch.

In other to ensure easy interpretation of these results, according to different bioremediation strategies, the uncontaminated soil will be named as control (Table 1).

Table 1: Sample label for bioremediation set-up

Sample label	**Soil batches**
Sample A	Contaminated soil
Sample B	Contaminated soil + Goat faces
Sample C	Contaminated soil + Goat faces + Fertilizer
Sample D	Uncontaminated soil
Sample E	Uncontaminated soil + crude oil
Sample F	Uncontaminated soil + crude oil + fertilizer
Sample G	Uncontaminated soil + crude oil + fertilizer + Goat faces

Media Preparation

Nutrient Agar for Total Heterotrophic bacteria, Sabouroud Dextrose Agar of Titan Biotech limited was used for the isolation of total fungal. Oil agar medium was prepared according to the modified minimal salts medium (MSM) composition of mills *et al.* (1978). The composition of this media was $MgSO_4.7H_20$ (0.42g), KCl (0.29g), kH_zP0_4 (0.83g), Na_2HP0_4 (1.25g) $NaN0_3$ (0.42), agar (20g), 1L of distill water. The media was prepared by adding 1% of crude oil to this mineral salt medium. The medium was used for the isolation of total petroleum utilizing bacteria. The medium was mixed thoroughly and autoclave at 15psi at 121^0c for 15mins and it was allowed to cooled to 45^0c and was aseptically poured into Petri-dishes .

Culturing, Isolation and Enumeration of Total Fungi

Isolation and enumeration of total fungal was done by serial dilution, sterile saline i.e 0.85% of sodium chloride was used as diluents for inoculums preparation. 1.0g of soil sample was aseptically transferred into a sterile test tube containing 9.0ml of the diluent. This gave 10^{-1} dilution subsequently 10^{-6} serial solutions were prepared from the 10^{-1} dilution. Then, 0.1ml aliquot of 10^{-6} dilution of each soil sample was aseptically removed with a sterile pipette and separately spread plated with flame sterilized glass spreader on Sabouroud dextrose agar plates. The cultured

plates were incubated at 35^0c for 5days. After incubation, the colonies appeared on the Sabouroud dextrose agar plates were recorded as counts of total fungi for all seven soil samples.

Isolation and Enumeration of total Hetrotrophic Bacteria

For isolation of total heterotrophic bacteria, nutrient agar medium was used. Nutrient agar plates was inoculated in quintuple with 0.1ml aliquots of 10^{-6} dilutions of each soil sample and incubated at 35^0c for 24hours. Colonial that appeared on the nutrient agar plates was counted and recorded as the count of total heterotrophic bacterial for all seven soil samples.

Isolation and Enumeration of Petroleum Utilizing Bacteria

For the isolation of the total heterotrophic degrading bacteria, oil agar medium was used. The oil agar plates were inoculated in quintuple with 0.1ml aliquots of 10^{-6} dilution of each soil samples and incubated at 35^0C for 7 days. Colonies appeared on the agar plates was counted after a week and resulted as the count of total heterotrophic degrading bacteria for the seven soil samples. The colonies counted were expressed as the colony forming unit (CFU) per gram soil.

Identification of bacterial isolates

The cultural, morphological and biochemical characteristics of the discrete bacterial isolates were compared with the recommendation in Bergey's manual of determinative bacteriology (1994). The morphological and biochemical test include; gram staining, motility, catalase, oxidase, citrate utilization, hydrogen sulphide production, indole production, methyl red and Voges proskauer tests.

Identification of fungal isolates

The presence or absence of septa in the mycelium, type of spore, presence of primary or secondary sterigmata, and other microscopic characteristics as well as cultural characteristics were used in the identification of the fungal isolates of the biodegradation flask set up (Cheesbrough, 2006).

Moisture content

5g of each soil batches were weighed in a wash glass and were hot dried in an ovum. After trying the soil, it was transferred immediately in a desicator to cool, and the soil was reweighed. The differences in weight in the unsterilized soil and the sterilized soil samples was taken as the moisture content of the soil as shown in table 2.

Stock Solution

Ten percent glycerol solution was prepared dispensed in McCartney bottles

and autoclaved at 121°C for l5minutes, allowed to cool, then the pure cultures were inoculated into each McCartney bottle, until the clear colourless solution turns turbid and were stored in the refrigerator. This served as storage medium for pure cultures for subsequent characterization.

Results and Discussion

The result in table 2, shows the differences in the moisture content of the different experimental set-up, indicating sample G (Uncontaminated soil + crude oil + fertilizer + Goat faces) having the highest moisture content while sample D (uncontaminated soil) has the lowest. This study has shown the effects of different types of organic nutrients supplements and crude oil on the moisture of the affected soil. The moisture content of NPK fertilizer supplements were higher, this could be due to its hygroscopic and deliquescent characteristics while fresh goat faces used in the study has inherent somewhat high moisture content. These attributes (*high moisture content*) enhances the growth of microorganisms at day 20 (fig. 1-6) which was evident in their higher percentage bioremediation.

Table2: Moisture Content of different soil samples

Sample label	**Soil batches**	**Unsterilized soil**	**Sterilized soil**	**Moisture content**
Sample A	Contaminated soil (control)	5g	4.7g	0.3
Sample B	Contaminated soil + Goat faces	5g	4.4g	0.6
Sample C	Contaminated soil + Goat faces + Fertilizer	5g	4.g	1
Sample D	Uncontaminated soil (control)	5g	4.8g	0.2
Sample E	Uncontaminated soil + crude oil	5g	4.6g	0.4
Sample F	Uncontaminated soil + crude oil + fertilizer	5g	4.2g	0.8
Sample G	Uncontaminated soil + crude oil + fertilizer + Goat faces	5g	3.8g	1.2

The effect of time on petroleum degradation was significant, as the petroleum degradation rate decreased with increase in time. This was evident in the result for total heterotrophic bacteria, total fungi and total petroleum degrading bacteria (fig. 2-7). The microbial counts were generally higher in the composite soil samples of the contaminated soil (contaminated soil plus goat faeces, contaminated soil plus goat faeces and fertilizer) than in soil samples of the control.

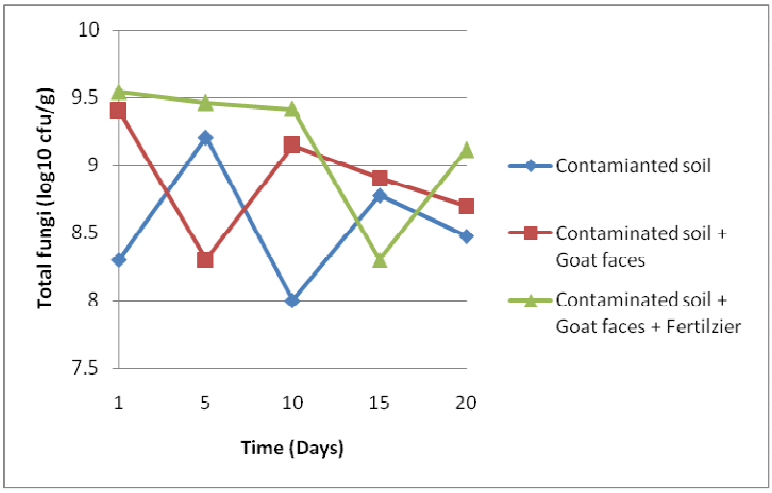

Figure 4: Total Fungi (log10 cfu/g) count during bioremediation of contaminated soil samples

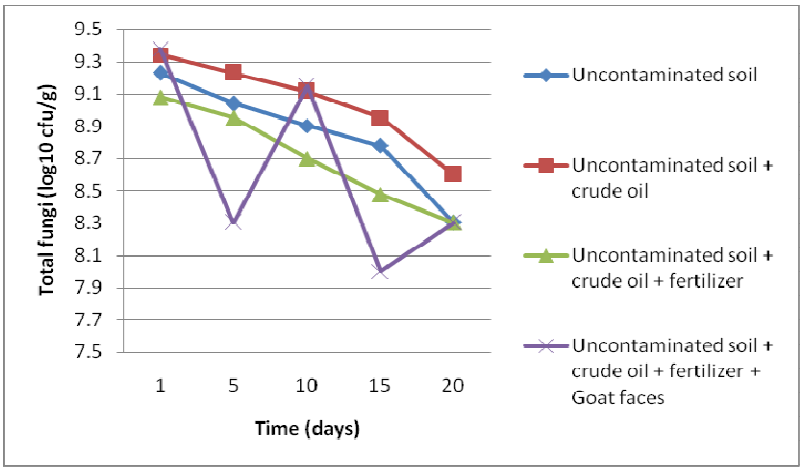

Figure 5: Total Fungi (log10 cfu/g) count during bioremediation of crude oil contaminated soil samples

Total fungal count and Petroleum Utilizing Bacterial count decreases with increase in time (days) comparatively (figure 4-7). This is because, on the first day, there were suitable feeding materials available for these microorganisms to feed on, but with increasing time, the lack of organic matter appeared little by little limiting the growth of the microorganisms. Shang-Hwan *et al.,* (2007) made a similar observation and concluded that hydrocarbon microbial population increased rapidly on the first day of 20 days testing period. They proposed these findings may be considered as an indicator for the feasibility study of oil contaminated soil bioremediation.

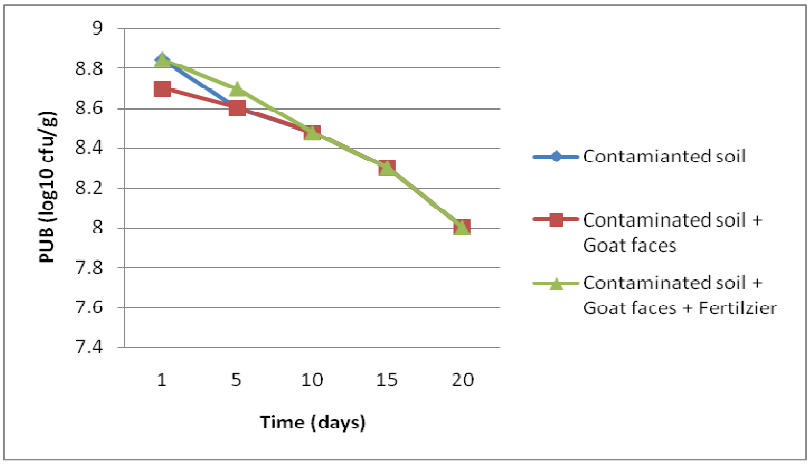

Figure 6: Petroleum Utilizing Bacteria (Cfu/g) during bioremediation of contaminated soil samples

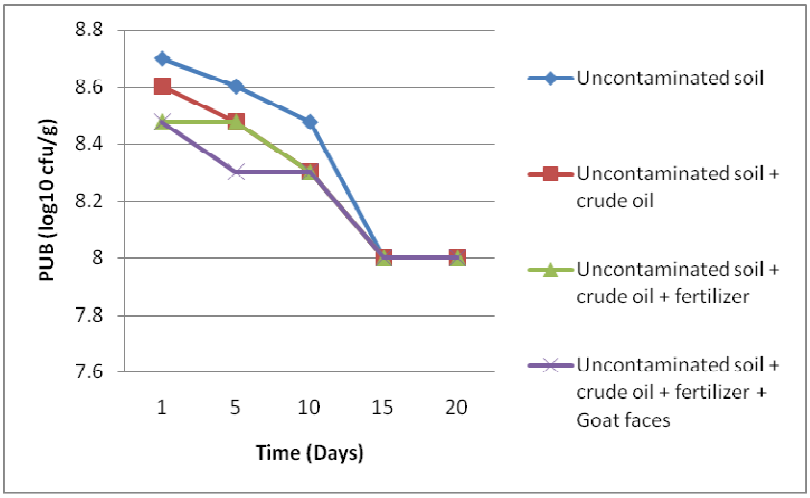

Figure 7: Petroleum Utilizing Bacteria (Cfu/g) during bioremediation of crude oil contaminated soil samples

The three genera of bacteria identified in this study were; *Pseudomonas* sp., *Bacillus* sp. and *Arthrobacter* sp. with *Pseudomonas* sp.and *Bacillus* sp. having highest occurance followed by *Arthrobacter* sp. Gogoi *et al.,* (2003), confirmed that the growth of *Arthrobacter* sp. were depressed by the presence petroleum hydrocarbon while it enhances the growth of *Pseudomonas* sp.and *Bacillus* sp. which they identified as the major users of diesel oil.

The characterization and of fungal isolates, which was based on morphological and microscopic features were of the genera; *Penicillium* sp., *Aspergillus* sp.,

Mucor sp. *and Rhizopus* sp.

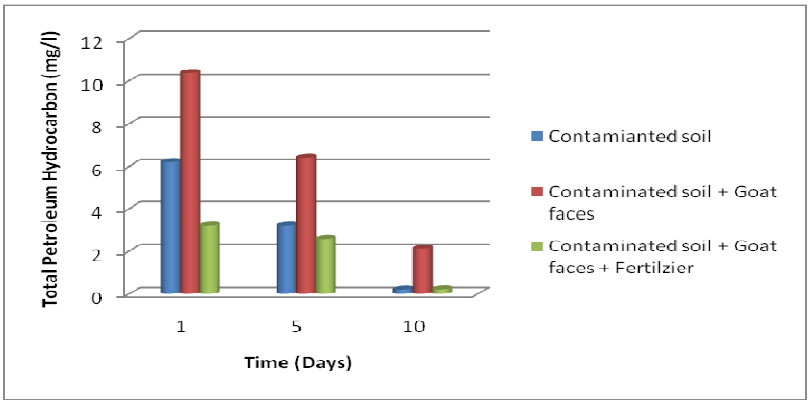

Fig. 8 Total Petroleum Hydrocarbon (mg/l) during bioremediation of contaminated soil

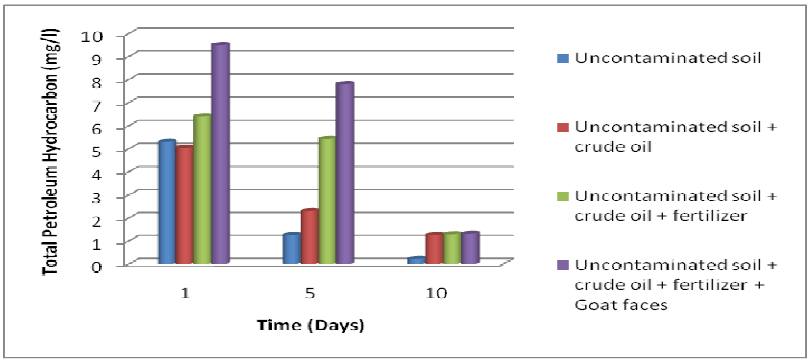

Fig. 9 Total Petroleum Hydrocarbon (mg/l) during bioremediation of soil contaminated with crude oil

In fig. 8 and 9, shows significant difference between soil treated with nutrient supplyment and the untreated soil samples. Statistically, oil degradation in the *lack* of treatment samples acting as control was less (uncontaminated soil 5.30mg/l, contaminated soil 6.20mg/l) and the oil degradation ratein the treatment samples with goat feces was higher (10.40mg/l) than the treatment sample with fertilizer (5.43mg/l). It was observed that between the oil degradation average in soil in both extraction time (1 to 10 days), there is significant differences. Shang-Hwan *et al.,* (2007) reported similar results that the initial level of oil in oil contaminated soil was reduced in the fertilized and goat feces soil. The lack of organic feeding matters, will limit the oil degradation and the untreated soil ratio will increase. This observation corresponded with the growth results of the microbial count. However, it was observed that *fresh* goat feces having high moisture content extended the nutrient depletion time; as such at day 15, contaminated soil with goat feces has high bioremediation rate (21.01%) Fig.10, table 3.

Table 3: Total Petroleum Hydrocarbon (TPH mg/l) percentage remediation rate.

Sample label	Amount of hydro-carbon remediat-ed (mg/L)	Percentage (%) rate of bioreme-diation
Contaminated soil	5.97	15.16
Contaminated soil + Goat faces	8.27	21.01
Contaminated soil + Goat faces + Fertilizer	2.96	7.52
Uncontaminated soil	5.07	12.88
Uncontaminated soil + crude oil	3.8	9.65
Uncontaminated soil + crude oil + fertilizer	5.1	12.95
Uncontaminated soil + crude oil + fertilizer + Goat faces	8.18	20.78

Ayotamuno *et al.,* (2006) showed that between the remediation periods there exists a negative relationship. However, in this present study, in the first day, because of a suitable environmental conditions and appropriate feeding, oil degradation was high, but in day five, the lack of nutritional elements caused the decrease of bioremediation process especially in those untreated soil. The treated soil shows a continuous phase of remediation, this could be due to nutrient and acclimatization of the degraders.

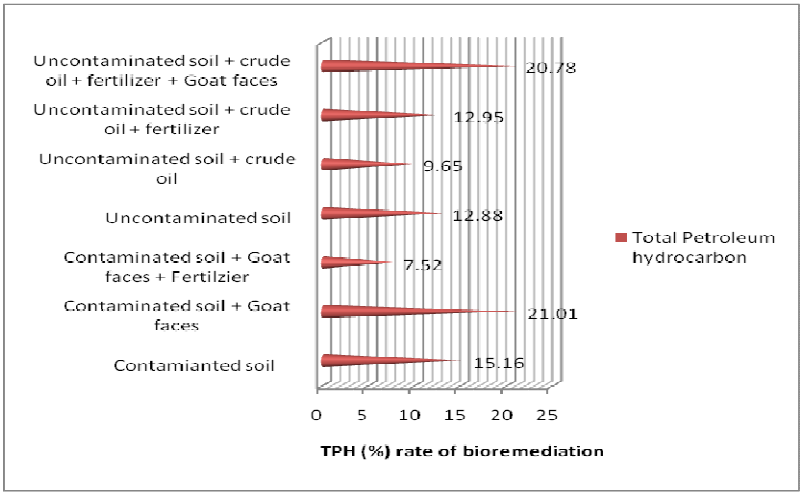

Fig. 10 Percentage (%) remediation of Total Petroleum Hydrocarbon (TPH)

Evaluating the actual amount of petroleum hydrocarbon degraded during the study, it shows that contaminated soil + goat feces have the highest (8.27mg/l) while uncontaminated soil + crude oil was lowest (3.80mg/l) table 2. The percentage (%) rate of

bioremediation reveals the following; Contaminated soil + goat feces (21.01%) > Uncontaminated soil +crude oil + fertilizer + goat feces (20.78%) > Contaminated soil (15.16%) > Uncontaminated soil +crude oil + fertilizer (12.95%) > Uncontaminated soil (12.88) > Uncontaminated soil +crude oil (9.65%) > Contaminated soil +crude oil + fertilizer + goat feces (7.52%).

Conclusion and Recommendation

Petroleum hydrocarbon pollution is a worldwide threat to the environment and the remediation of oil contaminated soils is a major challenge for environmental research. The combination of biostimulating agents' treatment strategies; NPK fertilizer and goat feces showed the ability to enhance petroleum hydrocarbon biodegradation, with goat feces treatment having greater petroleum hydrocarbon reduction than NPK fertilizer. Thus, an effective and environmental friendly method which is cost effective is the best option via the use of goat feces. *Fresh* goat feces extend the nutrient depletion time; thereby having a greater bioremediation potential more than other nutrient supplements. Thus, the moisture content of organic biostimulating agent is a factor to be taken into consideration.

Petroleum Utilizing Bacteria and fungi identified were; *Pseudomonas* sp., *Bacillus* sp. and *Arthrobacter* sp.; *Penicillium* sp., *Aspergillus* sp., *Mucor* sp. *and Rhizopus* sp. Microbial counts showed decrease in population with increasing time. This present work showed greater effectiveness goat feces than NPK fertilizer, high moisture content in the *fresh* goat feces extends the nutrient depletion time of organic biostimulating agents thus enhancing bioremediation. It is therefore recommended that companies and refineries whose activities contaminate soil with petroleum products should be encouraged to simply seed contaminated sites with *fresh* goat feces.

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