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Original

Degradation of Total Petroleum Hydrocarbon（TPH）in Contaminated Soil Using *Bacillus pumilus* MVSV3

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A study on bioremediation of soil contaminated with petroleum sludge was performed using *Bacillus pumilus*/MVSV3（Accession number JN089707）. In this study, 5 kg of agricultural soil was mixed well with 5% oil sludge and fertilizers containing nitrogen, phosphorus and potassium-（N:P:K）. The treatment resulted in 97% removal of total petroleum hydrocarbon（TPH）in 122 d in bacteria mixed contaminated soil when compared to 12% removal of TPH in uninoculated contaminated soil. The population of the microorganism remained stable after introduced into the oil environment. The physical and chemical parameters of the soil mixed with sludge showed variation indicating improvement and the pH level decreased during the experiment period. Elemental analysis and Gas Chromatography-Mass Spectroscopy（GC-MS）analysis revealed the bacterial ability to degrade oil sludge components. Growth experiments with *Trigonellafoenum-graecum*（Fenugreek）showed the applicability of bioremediated soil for the production.

Key words：Bioremediation / Biodegradation / Petroleum oil sludge / Contaminated soil.

INTRODUCTION

Crude oil is the feedstock for petrochemicals; these are exhaustible but still the dominant source of energy. During the process of refining crude oil, waste products, which are least degradable, are produced and are termed as oily sludge. This oily sludge, which is generated in the refinery on periodic cleaning of inlet and outlet tanks, causes environmental issues as well as are of human health concern. The leading problems faced by the refinery are the safe storage and disposal of the oily sludge. In India, earlier, petroleum was totally imported. Today 52% of India's crude oil is produced indigenously. The government owns the majority of refineries in India, and the total capacity of existing refineries till date is approximately 73 million tons/annum. Three types of sludge's are generated from the refineries; they are termed as oily sludge, bio sludge and chemical sludge. About 28,220 tons of total sludge quantity is produced every year in India（Bhattacharya and Shekdar, 2003）.

Many methods are followed to decontaminate this sludge before disposal. Bioremediation is the most often adopted method among all and is a known state-of-art technique, which employs the use of microorganism mostly in pure or as consortium. These organisms used can be indigenous, acclimatized or manipulated such that they utilize the organic compounds and other pollutants with ease. Bioremediation using such organism are comparatively more accepted by the public and are relatively low cost, that could also be carried out both in situ and ex situ. Mixing of fertilizers（N:P:K）, oil dispersant, surfactants and other bulking agents with the soil influences the speed as well the availability of the pollutant to the microbes（Macnaughton et al., 2003; Ramachandran et al., 2004; Mishra et al., 2001, NRC/NAS-National Research Council of the National Academy, 2005）.

Collection, treatment, transport and disposal of the sludge impose a financial burden too. Improper sludge management creates the high health risk to humans and adverse effect on the environment. Many studies on land farming have been carried out in a number of countries like USA（Kaplan and Kitts, 2004）, Italy（Zucchi et al., 2003）, Israel（Rosenberg et al., 1992）, India（Mishra et al., 2001）and Nigeria（Adams and

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Jackson., 1996). Bioremediation of the oil sludge in soil also known as land spreading (or land farming) is currently carried out in many parts of the world. It involves the application of the pollutant in known volume to soil and tilling it with N:P:K or other sources along with microbes or plants.

A local baseline technique for the efficient degradation of petroleum oil sludge by mixing a known quantity of it was carried out in this study. Prior to this studies lab scale experiments on optimum inocula to be used for availability of the substrates were conducted. Although many microbes, including consortium have also been studied for bioremediation, literature on degradation capacity of *Bacillus pumilus* is less available. Hence, this study seeks to investigate the efficiency and degrading potential of *B. pumilus* MVSV3 in the remediation of soil artificially contaminated with the petroleum sludge. The Total Petroleum Hydrocarbon (TPH) degradation rate was calculated. After several weeks of bioremediation of soil, growth of *Trigonellafoenum-graecum* (Fenugreek) was also monitored.

MATERIALS AND METHODS

Collection of materials

The petroleum oily sludge is formed during the production, transportation and storage process in the refinery plant. In this study, oil sludge was collected from the open-to the-air storage tanks of the country refinery (India). The samples were preserved at low temperature till the experiment commenced. Uncontaminated agricultural soil and the fertilizer-N:P:K at the concentration of 10:5:5 (fertilizer-10N:5P:5K) was obtained from a local agricultural nursery, Avadi (Chennai, India).

Preparation of experimental set up

For the degradation study of oil sludge, 5 kg agricultural (uncontaminated) soil was mixed well with 5% of oil sludge. The microorganism for seeding oily sludge polluted soil was added at predetermined amount; the bacteria *B. pumilus* MVSV3 (Accession number JN089707) was obtained from the stock culture. Based on initial liquid and soil studies on optimization of process parameters, it was determined that 2.6×10^{10} CFU/g of inoculum concentrations could be tested against the degradation studies of oil sludge in soil. The fertilizer-10N:5P:5K was applied once a week for one month, and then it was applied once in two weeks for 2^{nd}, 3^{rd}, 4^{th} months (Nkeng et al., 2012). The bacterial counts after the degradation period was determined by standard plate count method on nutrient agar plates and the colony forming units were counted after 24 h incubation at 30℃.

The experiments were carried out in 3 different trays. Agriculture soil was sterilized before the experiments to ensure no interference from other microorganisms (Anuradha et al., 2014). Tray A containing the unpolluted agricultural soil, tray B containing agricultural soil mixed with oil sludge, and these were inoculated with the microorganism. Tray C containing the agricultural soil was polluted well with oily sludge. Soils from these trays are further used in germination trails after degradation studies.

Analysis of selected parameters

Composition of the petroleum oily sludge was analyzed initially. Tests for pH, moisture content, total organic carbon (TOC) were also carried out. The moisture content of samples were determined by heating them at 80℃, where the water layer gets separated from the sample. The TPH was extracted from the sludge sample by using solvents such as hexane, ethylene chloride and chloroform. Solvents are evaporated in a fume hood by gentle nitrogen streaming. After the solvent has completely evaporated, the TPH in each sludge sample was quantified. Scanning electron microscopy coupled with electron dispersive X-ray spectrum (SEM/EDX, Hitachi-3400N) was used to analyze the elemental composition of soil mixed with petroleum oil sludge, before and after biodegradation. GC-MS (Gas chromatography and mass spectroscopy, JEOL GC MATE II, USA) was also carried out to determine components by matching the retention time with authentic standards.

Germination trials using *Trigonellafoenum-graecum* (Fenugreek)

The study on efficiency of bioremediation was conducted under laboratory conditions, by setting up a model experiment with three shallow pots, in which *Trigonellafoenum-graecum* (Fenugreek) were tested for germination. Earlier investigations were reported on growth of fenugreek on chromium-contaminated soil (Dheri et al., 2007). Agriculture soil was sterilized before the experiments to ensure no interference from other microorganisms (Anuradha et al., 2014). The seeds were sown in each pots at a depth of 1 cm. Trails carried out were: pot D: Uncontaminated soil from tray A + Seeds; pot E: Treated Soil from tray B + Seeds; pot F: Untreated soil from tray C + Seeds.

RESULTS

The composition of the petroleum oil sludge used in this study is tabulated in Table 1, which showed a high solid content of 38.2%. The TPH content in pure petroleum oil sludge was estimated to be 68,900 mg/kg. The pH did not show appreciable changes in tray C, but decreased from 6.8 to 5.3 in tray B. The TOC was also

TABLE 1. Composition of petroleum sludge

Constituents	Concentration (% wt/wt)
Oily sludge	
Total Solid	38.2
Ash	4.1
Moisture	34.5
TOC	8.8
Total Petroleum Hydrocarbon (mg/kg)	
TPH	68900

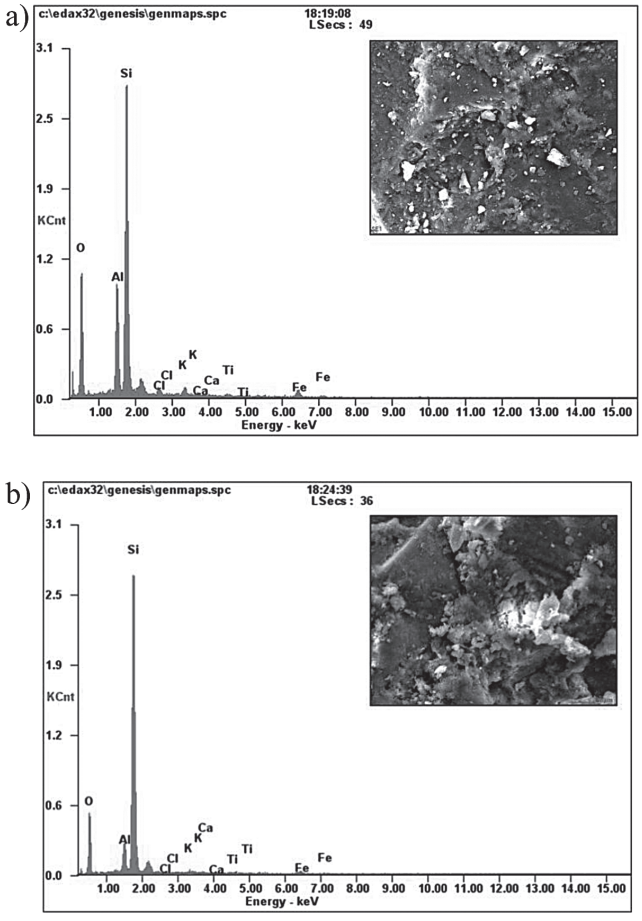

FIG. 1. SEM/EDX analysis of the contaminated soil in tray B (a) before and (b) after bioremediation [Conditions: Soil mixed with petroleum sludge + *B. pumilus* MVSV3, days of degradation - 0 d(before) and 122 d (after)]

observed to be decreasing from 8.8 to 1.5% in the soil sample from tray B due to bacterial activity.

The SEM/EDX analyses of the soil (Tray B) before and after degradation are represented in Fig.1. The elemental composition in terms of weight % is represented in Table 2. It is evident from the analysis that the soil before treatment from tray B is enriched with inorganic elements like aluminum, silicon, potassium, ferrous, titanium and calcium. Similarly, presence of high amount of silicon ions and traces of ferrous and aluminum are also found, which are normally found during the process of reclaiming of oil.

TABLE 2. EDX analysis of contaminated soil (Tray B) before and after degradation

Elemental composition	Before degradation Wt. %	After degradation Wt. %
O	41.26	34.89
Al	12.68	5.63
Si	55.96	38.49
Cl	1.02	0.01
K	1.64	0.49
Ca	0.46	0.23
Ti	0.55	0.24
Fe	3.91	2.57

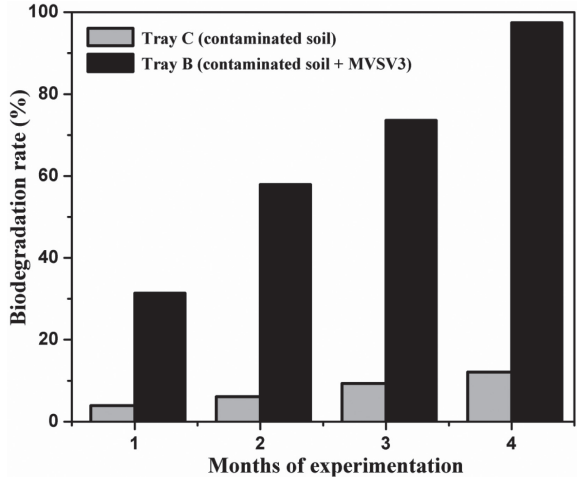

FIG. 2. Biodegradation rate (%) of TPH in soil [Conditions: Treated and untreated soil samples, Months of experimentation-1-4 months]

More amount of (Si) and (Al) compared to that of other elements indicates why the sludge soil shows resistance towards natural degradation. The decrease in elements like (Si), (O) and (Al) ions indicates the microbial mass increase and the ability of the bacteria in changing the elemental composition of the oil sludge mixed in soil during degradation.

The biodegradation rate (%) of petroleum oil sludge was monitored. Fig.2 indicates the biodegradation rate (%) of TPH in soil samples in tray B and C during the experiment. A degradation rate of 97% of TPH was recorded in the tray B (contaminated soil + MVSV3) against 12% (contaminated soil) in the tray C after 122

d. Survival of bacteria in contaminated soil after inoculation must be a major factor, which decides the degradation of TPH in liquid or soil (Ramos et al., 1991). At the end of the experiment, the bacterial count increased to 5×10^{14} cfu/g in tray B, indicating that *B. pumilus* MVSV3 could survive well in the contaminated environment, as they were isolated from petroleum-contaminated sites (Sugiura et al., 1996; Rahman et al., 2003).

The GC-MS analysis of soil mixed with petroleum oil sludge (control) and the biodegraded soil (test), showed difference in the composition of the component types. Typical components of gasoline like phenol, 2,4-bis(1,1dimethylethyl), 9-hexadececenoic acid-methyl ester, 1-eicosene, pentadecanoic acid-14-methyl-methyl ester, 8-octadecenoic acid-methyl ester, 3-heptadecene(Z), 1-hexacosene, hexadecane, heptadecane-7-ethyl, octadecene(E), benzenepropanoic acid- 3,5-bis(1,1-dimethylethyl)-4-hydroxy, heptadecanoic acid-15-methyl-methyl ester, 1-docosene and cyclotetracosane were identified in the control (Table 3a). Among them, compounds such as octanes and nonanes are usually degraded first because of their low molecular weight (Ramadan et al., 2012). However many peaks disappeared, newer peaks were found (Table 3b) and few peaks varied only in their peak intensity viz., pentadecanoic acid-14- methyl-methyl ester and 1-eicosene (Figure 3a-3b). Similar results were reported when *Candida viswanathii* was used for degrading poly aromatic compounds (Hesham et al., 2009) and diesel blend (Junior et al., 2009). This indicated the utilization of petroleum compounds by *B. pumilus* MVSV3 to form new intermediates. Comparison of the chromatograms also showed that C_{14}-C_{24} aliphatic was degraded to considerable extent to simpler compounds (Verma et al., 2006).

For germination trails the soil samples in trays were tilled well and was transferred to three shallow pots, namely pot D-: agricultural soil, pot E-: treated contaminated soil and pot F-: untreated contaminated soil. The pots were watered generously for the first few days and throughout the growing period. The sprouting time of the fenugreek seeds was supported by agricultural soil as well as by treated contaminated soil. Sprouting of *Trigonellafoenum-graecum* seeds occurred in 6 d in pot D, while it took 8-9 d in pot E. A significant growth of the seedling in pot E was observed when compared to complete inhibition in pot F even after 30 d, which is represented in Fig.4. The results of these trails suggested that the microorganism was not only effective in bioremediating the contaminated soil but also functioned in a long duration and helped in restoring some of the soil habitat that helped the seedlings to grow.

TABLE 3a. Compounds identified by GC-MS in contaminated soil (Tray B) before degradation by *B. pumilus* MVSV3

Compound	Retention time (min)
Phenol, 2,4-bis(1,1 dimethylethyl)	12.57
1-Hexadecanene	13.37
3-Heptadecene(Z)	14.62
Heptadecane, 7-Methyl	15.12
2-Octadecene(E)	15.73
9-Hexadececenoic acid, methyl ester	16.88
Pentadecanoic acid, 14-methyl, methyl ester	17.18
Benzenepropanoic acid, 3,5-bis(1,1-dimethyl-ethyl)-4-hydroxy	17.33
1-Eicosene	17.83
8-octadecenoic acid, methyl ester	18.87
Heptadecanoic acid, 15-methyl-, methyl ester	19.12
1-Docosene	19.77
Cyclotetracosane	21.55
1-Hexacosene	23.82

TABLE 3b. Compounds identified by GC-MS in contaminated soil (Tray B) after degradation by *B. pumilus* MVSV3

Compound	Retention time (min)
3-Oxa-6-thia-2,7-disilaoctane, 2,2,7,7-tetramethyl	12.17
2-Amino-2 – oxo-acetic acid	14.25
Tridecanoic acid	15.00
1-Octadecene	15.68
Pentadecanoic acid, 14-methyl-, methyl ester	17.18
5 (P aminophenyl)-4-octolyl-2-thiozolamine	17.63
1-Eicosene	17.78
Octadec-9-enoic acid	19.12
1-Phenazinecarboxylic acid (1-methoxyethyl)–methyl ester	20.23
2,7-Diphenyl 1-6 dioxopyridazinol	21.73
1, Benzopyran-4-one, 5-ethoxy-2-(3-ethoxy-4-methoxyphenyl)-7-methoxy	23.28
Propanoic acid 2,3 acetoxy 4,4,14-trimethylandrost	25.45

DISCUSSIONS

The efficiency of degradation of oil sludge was determined based on the changes in chemical and physical properties of the contaminated soil. The increase in biomass, decrease in pH and decrease in TPH indicated that the bacterium was capable of utilizing the oil sludge as carbon source. In this study, the deremedia-

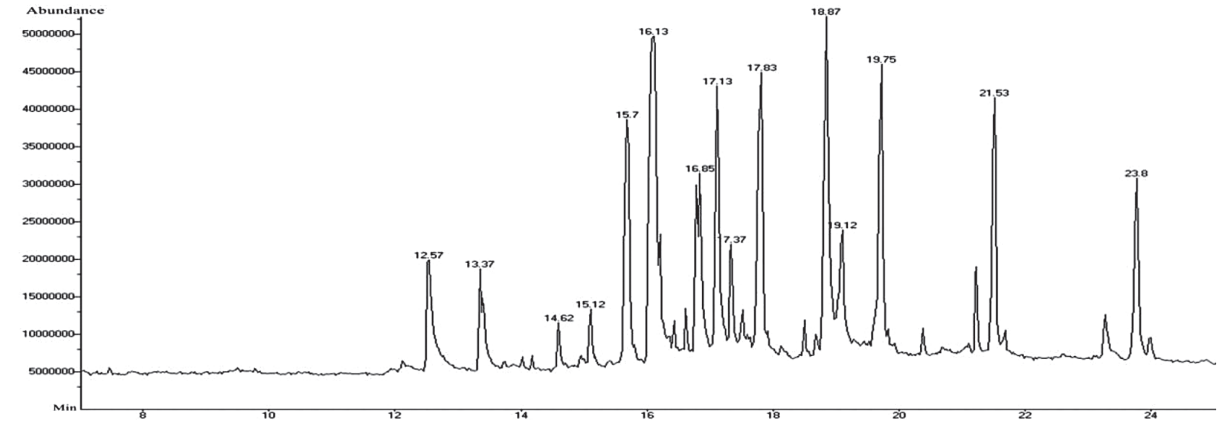

FIG. 3a. GC-MS of contaminated soil from tray B before (0 d) degradation

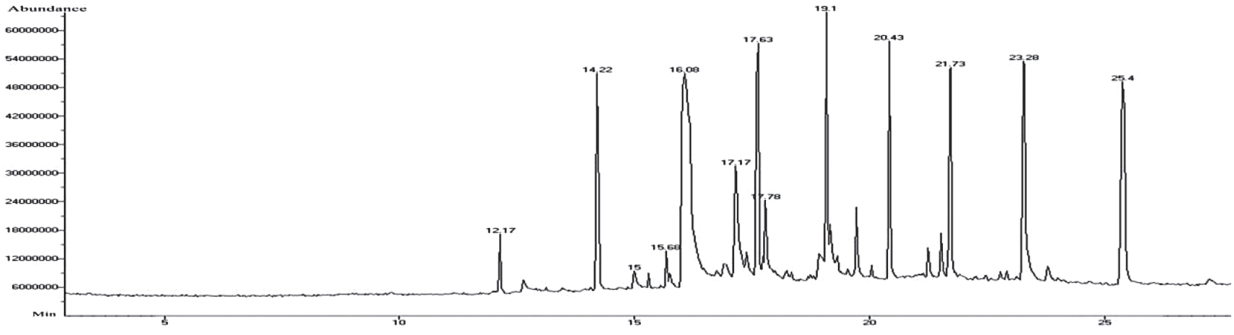

FIG. 3b. GC-MS of contaminated soil from tray B after (122 d) degradation

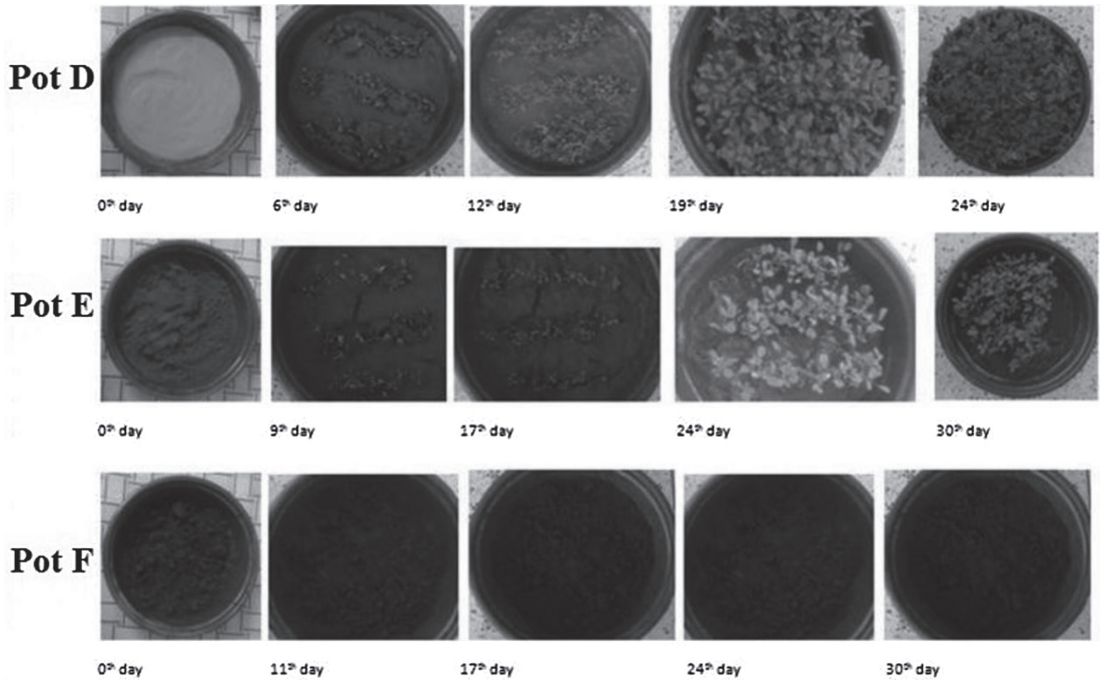

FIG. 4. Seed germination trails using *Trigonellafoenum-graecum* (*Fenugreek*).
[Soil samples after 122 d were used for germination tests].

tion of the TPH contaminated soil by pure culture of *B. pumilus* was carried out, and revealed the reduction of TPH in tray B to be higher (97%) than the tray C (12%). These patterns of TPH degradation were reported by Zucchi et al. (2003) and Vinas et al. (2005). And similar studies carried out by Cerqueira et al. (2011), demonstrated the degradation of oil sludge by five pure culture isolates viz., *Stenotrophomonas acidaminiphila*, *B. megaterium*, *B. cereus*, *B. cibi* and *Ps. aeruginosa*. Verma et al. (2006) also verified the degradation of oily sludge by three pure culture, *Bacillus sp*. SV9, *Acinetobacter sp*. SV4 and *Psuedomonas sp*. SV17. GC-MS analysis demonstrated that compounds like phenol, octadecanoic acid, hexadecane, heptadecanoic acid, docosene cyclotetracosane and some of their esters were preferentially degraded compared to eicosene and pentadecanoic acid. Petroleum oil sludge is a complex mixture of n-alkanes, asphalthenes, resins and aromatics. Microorganisms generally degrade a specific component in oil. There are many reports that has observed the same component being degraded to different extent by the same organism, which is suggested to be due to bioavailability of the compound in the oil sample (Sugiura et al., 1996). This study shows the ability of the bacteria to reduce the abundance of specific compounds and production of less complex, acidic compounds during degradation process in the time span of 122 d.

One of the major selective environmental factors that affect the microbial growth and activity, enzyme activity, nutrient availability and transport process is the pH (Dhote et al., 2010). The pH of the test tray B decreased by the end of the experiment. This can be attributed to the production of organic acids during hydrocarbon degradation. Janbandhu and Fulekar (2001) also found similar results when a microbial consortium was grown on phenanthrene, which showed a decrease of pH from 7.0 to 5.2. On the Contrary, a few authors reported an increase in pH value from 4.9 to 7.5 (Nkeng et al., 2012). This was suggested to be result of treating the oil sludge mixed soil with emulsifier. Addition of chemical fertilizers like N:P:K to soil also lead to higher production of organic acids, which further relates to the decrease in pH (Kincannon, 1972 and Amadi et al., 1996). The test tray when investigated for microbial community indicated a stable growth of microorganism even after 122 d, which is essential for TPH degradation. Vasudevan and Rajaram (2001) reported similar result after 90 d of oil degradation in soil.

Seed germination is a sensitive stage of growth in plants; this can be exploited to determine the effect of pollutants exposure (Banks and Schultz, 2005). The total seed germination after degradation experiment was determined. One hundred percent seed germination was seen in uncontaminated soil (pot D), while a decrease in number of seeds that germinated was seen in pot E. Due to heavy oil contamination, a negative effect was observed in the pot F, with no growth of seedlings. Adam and Duncan (2002) also observed reduced germination, in soil contaminated by petroleum and it's by products. Similarly, germination of Green gram, *Vigna radiate L* was studied on petroleum hydrocarbon contaminated soil (Masakorala et al., 2013). These results also indicated that pot E, had lower concentration of TPH in soil, which did not cause much inhibition on fenugreek seed germination unlike in pot F. Further, the adopted approach on trails of growing *Trigonellafoenum-graecum* provided a positive indication for its application in to larger field areas. Due to increasing contamination, toxic and carcinogenic nature affecting the human health and environment, the biotreatment of petroleum oil sludge using such competent bacteria provides additional information in this field.

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