Journal of Environment and Earth Science
ISSN 2224-3216 (Paper) ISSN 2225-0948 (Online)
Vol. 3, No.11, 2013
www.iiste.org

Identification of Lubricating Oil-Degrading Microorganisms in Oil Polluted Soils from Five Auto- mechanic Workshops in Accra, Ghana

E. Ameko*, S. Achio, S. Alhassan and I. Sadique
Department of Science Laboratory Technology, Accra Polytechnic , Accra, Ghana. Box 561, Accra, Ghana
* E-mail of the corresponding author: edmundameko@yahoo.com

Abstract
Trichothecium, Trichoderma, Aspergillus niger, Fusarium, and *Penicillium spp*. from oil contaminated soil from 5 Mechanic Shops in Accra, Ghana showed zones of clearance of oil on Minimum Salt Enrichment Medium (MSEM) Agar seeded with 1000ppm Engen™ Lubricating Oil (ELO), so were counted as presumptive lubricating oil-utilizing moulds. Significant increases ($P \geq 0.05$) in viable counts, fungal dry weights and optical densities; significant decreases ($P \leq 0.05$) in pH's of pure cultures of the moulds in MSEM+1.0%(v/v) ELO medium at 30°C for 0 - 25 day's; positive correlations between viable counts and fungal dry weights, viable counts and optical densities, and fungal dry weights and optical densities; and negative correlations between pH and viable counts, and pH and optical densities, confirmed the moulds as lubricating oil consuming fungi with potential for use in bioremediation of oil polluted soils. *Aspergillus niger* exhibited the highest bioremediation capacity and *Trichothecium* the least.
Keywords: Lubricating Oil, Pollution, Fungi, Bioremediation

1. Introduction

Ghana may pump as much as 240,000 barrels of oil from its offshore Jubilee field by 2014-15. Even before Ghana began commercial production of its first oil in 2011, there were major spillages of oil. In December 2009, Kosmos spilled about 600 barrels of low toxicity oil-based mud in its exploratory operations in the Jubilee Fields. Another spill occurred in March 2010 (The Ghanaian Times, 2010).

There are various methods of containing oil spills and these can be grouped into three, and these are Physical, Chemical and Biological methods respectively. Physical methods include incineration and sinking the oil. Incineration may destroy indigenous organisms, including oil-degrading microbes, and increase the toxicity of the petroleum residue. Sinking the oil with heavy hydrophobic agents such as ground chalk merely removes the oil to anaerobic sediments or deep ocean floor, where long persistence of the oil pollutant is bound to occur (Nwangwu and Okoye, 1981). Chemical methods for removing or dispersing spilled oil from the environment have side-effects on the ecosystem and are toxic, which is sometimes more pronounced than that of the oil itself (Vogt, 2010). Biological agents in the form of fungi (Sood and Lal, 2009) and bacteria contain the spill by enhancing biodegradation of the oil into harmless substances such as water and carbon dioxide (Bioremediate.com, 2010), which could result in restoring oil-contaminated environments. Microbial degradation has emerged as the most significant natural mechanism for the removal of non-volatile hydrocarbon pollutants from the environment. Although biodegradation occurs at a very slow rate, it can be enhanced by inoculation with more efficient oil degrading microorganisms, or by introducing air and nutrients into the environment (Bioremediate.com, 2010). Species of microorganisms are however habitat specific (Obire, 1988). Filamentous fungi, yeasts, actinomycetes and bacteria all have the ability to utilize hydrocarbon substrates—though their ability to do so varies among individual strains. A study by Westlake et al. (1974) showed that the effect of oil on microbial populations depends upon the chemical composition of the oil and on the species of microorganisms present. Populations of some microbes increase; typically, such microbes use the petroleum hydrocarbons as nutrients. The same crude oil can favor different genera at different temperatures. According to Obire (2009), Atlas and Bartha (1972) showed that some crude oils contain volatile bacteriostatic compounds that must degrade before microbial populations can grow. On the other hand, some microbial populations decrease or show a neutral response to petroleum hydrocarbons. The overall effects of petroleum hydrocarbons on total microbial diversity remain unclear.

Commercial fungal and bacterial inocula are used in the developing countries for bioremediation of oil spills and as a first step in developing indigenous and habitat specific microorganism for use in large scale oil spill response operations this project looked at the isolation and identification of oil degrading microorganisms from oil polluted soils of some mechanic shops in Accra.

2. Methodology

2.1. Collection of Soil samples

Soil samples (400 g) from surface soil (0-15 cm depth) was collected with an auger from 5 Mechanic Fitting Shops in Accra, Ghana, consecutively after tilling with a sterile scoop and transferred into sterile polythene bags. Soil sample collections were made from 3-4 random points per shop and then mixed to form composite soil samples. All microbiological analyses were carried out within 24 h after sample collection. All soil samples for future analyses were stored at 4 ± 2 °C according to ISO and OECD standards (Obire and Anyanwu, 2009).

2.2 Culturing of Microorganisms

Appropriate dilutions of soil samples were prepared and 0.1 ml aliquots cultured into Plate Count Agar for total viable count. Appropriate dilutions of soil samples were prepared and 0.1 ml aliquots cultured into nutrient agar by pour plate technique for growth of bacterial species. Fungi species were cultured using Potato Dextrose Agar (PDA) to which streptomycin (50 mg/ml) had been added to suppress bacterial growth. The colonies counted were computed and expressed as colony forming unit (cfu) per gram of soil.

2.3 Isolation of Microorganisms

Staphylococcus aureus and *E. coli* were isolated from the soil samples by sub-culturing 0.1 ml of appropriate dilutions of the soil samples into fresh Baird – Parker Agar and Eosin Methylene Blue Levine (EMB) Agar respectively, by pour plate technique. Positive controls of *Staphylococcus aureus* and *E. coli* respectively were used for the tests. Fungi were isolated using Dichloran Rose – Bengal Chloramphenicol Agar (DRBC) medium. The isolated cultures of bacteria and fungi were subsequently screened for the ability to utilise lubricating oil as sole carbon source.

2.4 Screening of Bacterial and Fungal Isolates for Their Ability to Utilise Lubricating Oil

2.4.1 Preparation of Minimal Salt Enrichment Medium (MSEM).

The method of Mills *et al* (1978) as modified by Okpokwasili and Okorie, (1988) was used in preparing a minimal salt medium (MSEM) of composition 6.0 g Na_2HPO_4, 3.0 g KH_2HPO_4, 0.5 g NaCl, 1.0 g HN_4Cl, 1.0 ml of 24.6% (w/v) $MgSO_4.7H_2O$ and 1.0 ml of 1.47% (w/v) $CaCl_2.2H_2O$ in 1000 ml distilled water), which was then dispensed into 250 ml conical flasks and sterilised. To each flask was added 1.0% v/v (i.e. 1.50 ml) Engen™ Lubricating Oil.

2.4.2 Preparation of MSEM Oil-Agar.

MSEM Oil-Agar was prepared by adding 1000 ppm Engen™ Lubricating Oil to molten agar prepared with MSEM solution and sterilising in an autoclave.

2.4.3 Culturing of bacteria on MSEM Oil-Agar.

Isolated bacteria were cultured in nutrient broth for 48 hours and 0.1 ml aliquots of appropriate dilution cultured into Oil-agar plates by pour plate technique.

2.4.4 Culturing of fungi on MSEM Oil-Agar.

Isolated fungi were cultured in malt extract broth for 48 hours and 0.1 ml aliquots of appropriate dilution cultured into Oil-agar plates by pour plate technique.

2.4.5 Preliminary tests of Isolates as Presumptive Lubricating Oil-Utilising Microorganisms.

Colonies which developed and showed growth of colonies and zones of clearance of oil on the MSEM oil-agar plates were counted as presumptive petroleum-utilizing moulds. Colonies which developed and showed growth of colonies and zones of clearance of oil on the oil-agar plates were identified by morphological and biochemical techniques for bacterial cultures and morphological characteristics and microscopy for Fungal isolates (Benson, 1990).

2.4.6 Tests to Confirm Microorganisms as true lubricating oil-utilising Microorganisms

2.4.6.1 Preparation of Minimal Salt Enrichment Medium plus lubricating oil.

A 10 ml volume of MSEM medium plus 1.0% v/v (i.e. 0.1 ml) Engen™ Lubricating Oil was dispensed severally into test tubes and sterilised (Okpokwasili and Okorie, 1988).

2.4.6.2 Culturing of Microorganisms.

The sterilised MSEM-Oil medium in the test tubes were inoculated with the pure cultures of Presumptive Lubricating Oil-Utilising Microorganisms and placed in an incubator (QL Model 10 – 140) at 30°C.

2.4.6.3 Measurements for Confirming the Utilisation of lubricating oil.

Utilisation of lubricating oil was monitored for incubation periods of 0, 2, 6, 13, 21 and 25 days, by measuring fungal growth, fungal dry weight, optical density and pH.

2.4.6.3.1 Fungal growths.

Fungal growths were measured by obtaining the viable counts on nutrient agar. The fractional change in viable count for each incubation period was obtained from the formula

$(V_n – V_0) / V_0$

Where V_n is the viable count for incubation period n days and V_0 is the viable count for incubation period of 0 days.

2.4.6.3.2 Optical density (OD).
Optical density was determined at 600 nm wavelength with Unico UV 2100 spectrophotometer by measuring the percent transmittances (%T) which were then converted to optical densities using the formula: OD = 2 – log of percent transmittance (Benson, 1990).
The fractional change in optical density for each incubation period was obtained from the formula
$(OD_n – OD_0) / OD_0$
Where OD_n is the optical density for incubation period n days and OD_0 is the optical density for incubation period of 0 days.
2.4.6.3.3 Fungal dry weight (FD).
Fungal dry weight was determined by drying Whatman No. 1 filter paper in a hot air oven at 105^{o}C to constant weight (W_c) and then harvesting the fungi on the filter paper by filtration, drying the filter paper overnight in the oven at 105^{o}C, and obtaining the dry weight (W_d) of the filter paper again on a digital weighing balance (ADAM AFA – 120 LC model).
The fungal dry weight (Fdw) was determined from the formula $W_d – W_c$.
The fractional change in fungal dry weight for each incubation period was obtained from the formula
$(FD_n – FD_0) / FD_0$
Where FD_n is the fungal dry weight for incubation period n days and FD_0 is the fungal dry weight for incubation period of 0 days.
2.4.6.3.4
The pHs were determined with an ACCULAB V-1200 pH meter.

3. Results and Discussion

Soils from all 5 oil contaminated sites contained various microorganisms and total viable counts ranged between 1.82 x 10^5 cfu/g – 2.76 x 10^5 cfu/g (Table 1), and the presence of *E. coli, S. aureus* and fungi in the soils were confirmed (Table 2).

Table 1. Total viable count (cfu/g) on Plate Count Agar (PCA) of microorganisms from oil contaminated soils from 5 Mechanic shops in Accra, Ghana

Soil Samples	Average (cfu/g)
Mech Shop 1	2.56 x 10^5
Mech Shop 2	1.82 x 10^5
Mech Shop 3	2.76 x 10^5
Mech Shop 4	2.10 x 10^5
Mech Shop 5	2.11 x 10^5

Table 2. Mean microbial counts (cfu/g) from oil contaminated soils from 5 Mechanic shops in Accra, Ghana. *S. aureus* was cultured on Baird – Parker Agar. *E. coli* on Eosin Methylene Blue Levine (EMB), and fungi on Dichloran Rose – Bengal Chloramphenicol Agar (DRBC).

Soil Samples	*Staph. aureus*	*E. coli*	*Fungi*
Mech Shop 1	4.8 x 10^2	19.3 x 10^2	8.3 x 10^2
Mech Shop 2	34.8 x 10^2	22.8 x 10^2	14.6 x 10^2
Mech Shop 3	19.4 x 10^2	10.9 x 10^2	13.1 x 10^2
Mech Shop 4	No growth	No growth	3.65 x 10^2
Mech Shop 5	8.4 x 10^2	No growth	16.0 x 10^2

For soils containing *E. coli*, numbers ranged between 1.09 x 10^3 – 2.28 x 10^3 cfu/g, and for *S. aureus* 4.80 x 10^2cfu/g – 3.48 x 10^3 cfu/g, and 8.30 x 10^2cfu/g – 1.46 x 10^3 cfu/g for fungi (Table 2).
In the preliminary tests for the Presumptive Lubricating Oil-Utilising ability of the isolates, the *E. coli*, and *S. aureus* colonies did not show any zones of inhibition on the MSEM oil-agar plates (Table 3). However, various fungal isolates showed zones of clearance on the MSEM Oil-agar plates which presumed that they had the ability to utilise lubricating oil as their sole carbon source (Table 3).
The Presumptive Lubricating Oil-Utilising fungi were identified to be *Trichothecium*, *Trichoderma*, *Aspergillus niger*, *Fusarium*, and *Penicillium spp*. (Table 4).
In petroleum-producing regions of Nigeria, Obire (1988) found several species of oil-degrading aquatic fungi in the genera *Candida, Rhodotorula, Saccharomyces* and *Sporobolomyces* (yeasts) and, among filamentous fungi, *Aspergillus niger, Aspergillus terreus, Blastomyces spp. Botryodiplodia theobromae, Fusarium spp. Nigrospora spp. Penicillium spp. chrysogenum, Penicillium spp. glabrum, Pleurofragmium spp.* and *Trichoderma harzianum*. Sebiomo et al (2010) isolated *Aspergillus flavus, A. niger, A. terreus, A. ochraceus,* and *Trichoderma*

spp. from oil contaminated soil from a mechanic workshop at Ago-Iwoye, Ogun State Nigeria which were capable of utilising lubricating oil as the sole carbon source for producing energy.

In this project, *E. coli* were identified in soils from 3 of the shops (Table 3), but they did not exhibit the ability to utilise lubricating oil as sole carbon source. Also, none of the *Staph. aureus* isolated from 4 out of the 5 sites were able to utilise lubricating oil as the sole carbon source.

Table 3. Colonies of microorganisms from oil contaminated soils from 5 Mechanic shops in Accra, Ghana which showed zones of inhibition on MSEM Oil-Agar.

Soil Samples	*Staph. aureus*	*E. coli*	Fungi
Mech Shop 1	NG	NG	GWZI
Mech Shop 2	NG	NG	GWZI
Mech Shop 3	NG	NG	GWZI
Mech Shop 4	NG	NG	GWZI
Mech Shop 5	NG	NG	GWZI

NG - No growth GWZI - Growth with zones of inhibition

Table 4. Fungi isolated from oil contaminated soils from 5 Mechanic shops in Accra, Ghana identified as Presumptive Lubricating Oil-Utilising Fungi. Culturing was on Dichloran Rose – Bengal Chloramphenicol Agar (DRBC)

Soil Samples	Fungi
Mech Shop 1	*Trichothecium, Trichoderma, Aspergillus niger*
Mech Shop 2	*Fusarium*
Mech Shop 3	*Trichothecium, Penicillium spp.*
Mech Shop 4	*Aspergillus niger, Penicillium spp., Trichothecium*
Mech Shop 5	*Aspergillus niger, Penicillium spp.*

The 5 fungi had adapted to living in the oil polluted environment occurring in the soils at the mechanic shops. The most common mould found in the soils was *Trichothecium*, followed by *A. niger*, and *Penicillium spp*. The least was *Trichoderma* in 10% of sampled soils (Table 5).

Table 5. Occurrence of lubricating oil utilising fungi in oil contaminated soils from 5 Mechanic shops in Accra, Ghana.

Microorganism	% of soil samples with the microorganism
Trichothecium	60%
Aspergillus niger	50%
Trichoderma	10%
Fusarium	20%
Penicillium spp.	50%

Akoachere et al (2008) isolated some oil-degrading bacteria from oil contaminated and uncontaminated soil samples and these were *Pseudomonas fluorescens, Serratia marcescens Bacillus mycoides, and Serratia,* but did not isolate *E. coli* or *S. aureus*.

Sebiomo et al (2010) isolated 10 bacterial species including *Pseudomonas stutzeri, P. putida, P. aeruginosa, P. mallei, Bacillus licheniformis, B. cereus, B. Subtilis, Corynebacterium spp. Alcaligenes eutrophus* and *Enterobacter aerogenes* from oil contaminated soil from a mechanic workshop Ago-Iwoye, Ogun State Nigeria which were capable of utilising lubricating oil as the sole carbon source for producing energy.

The profiles showing the fractional changes in viable counts (VC), pH, fractional changes in fungal dry weights (FDW), and fractional changes in optical densities (OD) as the 5 fungi utilised the lubricating oil for growth are shown in figures 1 to 5.

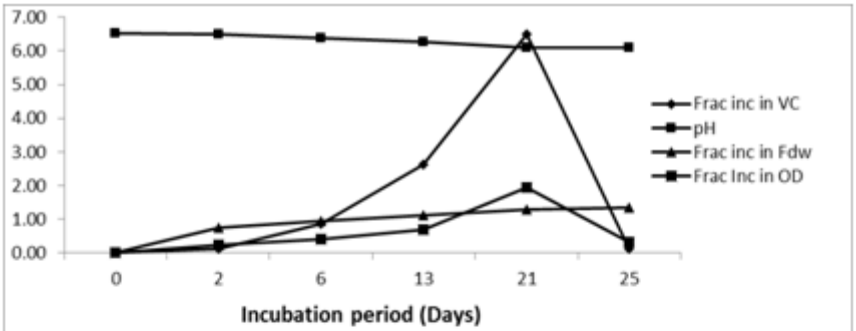

Fig 1. Growth profile of Trichoderma in Minimum Salt Enrichment Medium seeded with 0.1% (v/v) Engen ™ Lubricating Oil

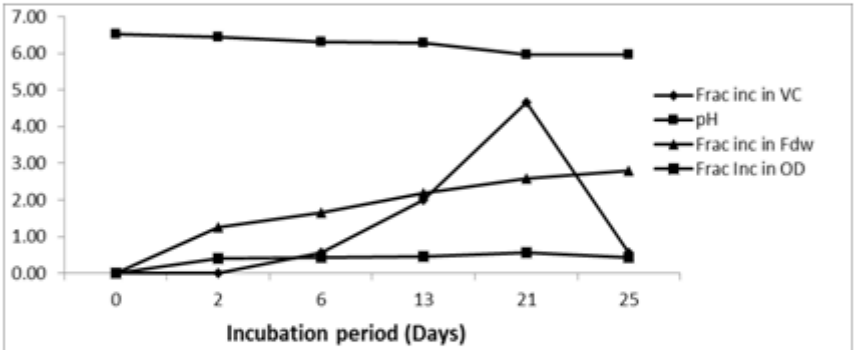

Fig 2. Growth profile of Fusarium in Minimum Salt Enrichment Medium seeded with 0.1% (v/v) Engen ™ Lubricating Oil

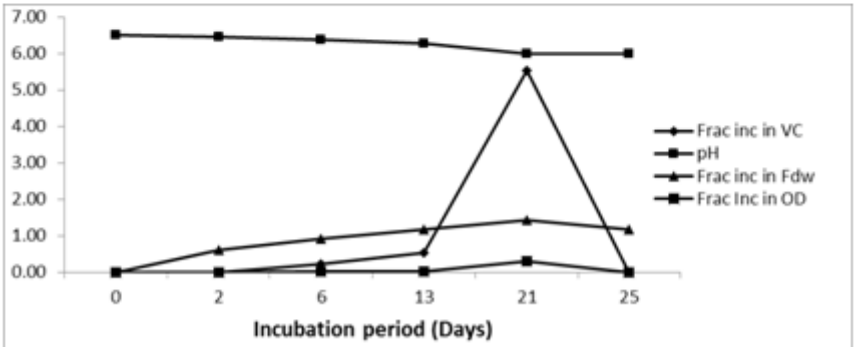

Fig 3. Growth profile of Trichothecium in Minimum Salt Enrichment Medium seeded with 0.1% (v/v) Engen ™ Lubricating Oil

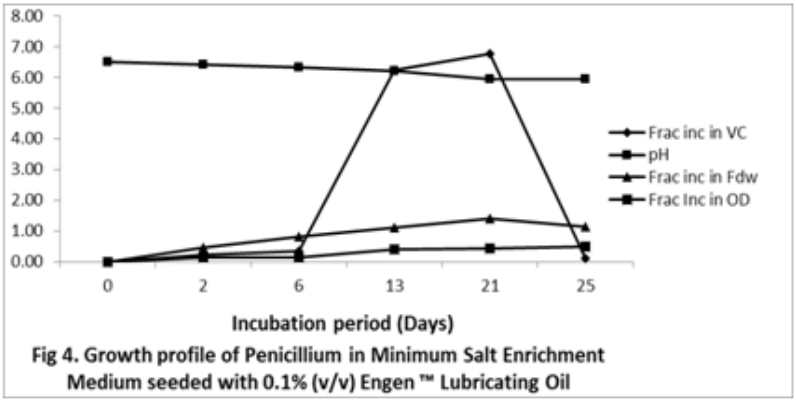

Fig 4. Growth profile of Penicillium in Minimum Salt Enrichment Medium seeded with 0.1% (v/v) Engen ™ Lubricating Oil

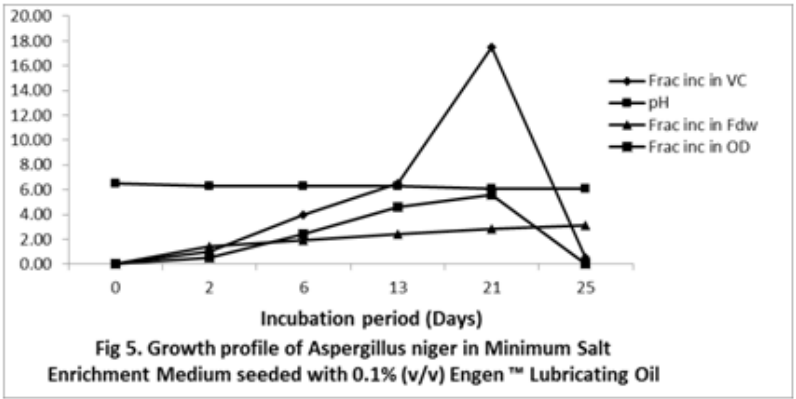

Fig 5. Growth profile of Aspergillus niger in Minimum Salt Enrichment Medium seeded with 0.1% (v/v) Engen ™ Lubricating Oil

There were increases in viable counts with increase in incubation period from day 0 – 21 (Figures 1 – 5) and these were significant ($P \leq 0.05$). There were decreases in viable counts from day 21 – 25. The increase in viable counts among the 5 fungi were significantly different ($P \leq 0.05$) with *A. niger* exhibiting the highest mean fractional increase of 5.80 over the incubation period of day 0 – 25, and *Trichothecium* with the lowest with 1.26 over the same period. This indicates that the fungi were able to utilise the lubricating oil as carbon source for growth and multiplication. However, from day 21 onwards it is possible that the accumulation of toxic metabolites in the closed system inhibited the further growth of the fungi. As more metabolites accumulated from day 21 – 25 there was decrease in fungal growth which could be due to feedback inhibition (Affenzeller et al, 1989).

There were significant decreases ($P \leq 0.05$) in the pHs over the incubation period but these decreases in pH's were not significantly different ($P \leq 0.05$) among the 5 fungi. During metabolism fungi produce acidic metabolites and as these metabolites accumulate the pH reduces (Punekar et al, 1985).

There were significant increases ($P \leq 0.05$) in fungal dry weight over the incubation period from day 0 – 25 (Figures 1 – 5). There were significant differences ($P \leq 0.05$) in the increased dry weights among the 5 fungi, with *Asp. niger* exhibiting the highest mean fractional increase of 1.73 over the incubation period of day 0 – 25, and *Penicillium spp*. with the lowest increase of 0.76, while *Trichoderma* and *Trichothecium* both had fractional increases in dry weight of 0.82 over the same period.

The optical densities showed significant increases ($P \leq 0.05$) over the incubation period from 0 – 25 days (Figures 1 – 5), with significant differences ($P \leq 0.05$) among the 5 fungi, with *A. niger* exhibiting the highest mean fractional increase of 2.61, followed by *Trichoderma* with 0.65, *Fusarium* 0.37, *Penicillium spp*. 0.23 and *Trichothecium* had the lowest of 0.07. As the fungi multiply the number of cells in the medium also increases and the medium becomes denser and the amount of light transmitted through the medium decreases.

There were positive correlations of (0.56) between viable counts and fungal dry weights, (0.81) between viable counts and optical densities, and (0.69) between fungal dry weights and optical densities respectively (Table 6).

Table 6. Correlations between pH, Viable count (VC), Fungal dry weight (FDW) and Optical density (OD) of 5 fungi cultured in MSEM+1.0%(v/v) ELO medium at 30°C for 0 - 25 day's

	pH and OD	pH and VC	VC and FDW	OD and VC	OD and FDW
Trichoderma	-0.66	-0.60	0.48	0.98	0.57
Fusarium	-0.67	-0.64	0.57	0.60	0.92
Trichothecium	-0.59	-0.59	0.57	0.99	0.56
Penicillium spp.	-0.93	-0.49	0.67	0.59	0.92
Asp. niger	-0.42	-0.53	0.49	0.90	0.49
Mean	-0.65	-0.57	0.56	0.81	0.69

The correlations between pH and viable counts, and pH and optical densities respectively were negative (-0.57) and (-0.65).

These results are similar to those of Sebiomo et al (2010) where negative correlations were obtained between optical density and pH, and between viable counts and pH, and positive correlation between viable counts and optical density for both bacterial and fungal isolates.

Conclusion

The results of this study indicate that the five fungi (*Trichothecium*, *Trichoderma*, *Aspergillus niger*, *Fusarium*, and *Penicillium spp*.) isolated from the oil contaminated soils from the mechanic workshops in Accra, are lubricating oil consuming fungi, and among them *A. niger* exhibited the highest lubricating oil utilising capacity, and the least was by *Trichothecium*.

References

Affenzeller, K., Jaklitsch, W.M., Hönlinger, C. and Kubicek, C.P. Lysine biosynthesis in Penicillium chrysogenum is regulated by feedback inhibition of α-aminoadipate reductase. FEMS Microbiology Letters, Volume 58, Issue 2-3, pages 293–297, April 1989. Article first published online: 27 MAR 2006. http://onlinelibrary.wiley.com/doi/10.1111/j.1574-6968.1989.tb03062.x/abstract

Akoachere, J. F. T. K., Akenji, T. N., Yongabi, F. N., Nkwelang, G., and Ndip, R. N. 2008. Lubricating oil-degrading bacteria in soils from filling stations and auto- mechanic workshops in Buea, Cameroon: occurrence and characteristics of isolates. African Journal of Biotechnology Vol. 7 (11), pp. 1700-1706, 3 June, 2008. http://www.academicjournals.org/AJB

Benson, H. J. 1990. Microbiological Applications: A Laboratory Manual in General Microbiology. 5th ed. McGraw-Hill Companies. pp. 34 – 39, 151 - 155

Bioremediate.com, Environmental Quality Assurance. 2010, Questions about Hydrocarbon Bioremediation. http://www.bioremediate.com/petrochemical.htmi

Harms H, Schlosser D, Wick LY. 2011. Mycology Research Today. Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. http://mycology.researchtoday.net/archive/7/2/2496.htm

McLure, J., 2010. Ghana Lacks 'Capacity' to Tackle Oil Spills. Bloomberg L.P. http://www.bloomberg.com/news/2010-10-08/ghana-lacks-the-capacity-to-tackle-spills-from-oil-wells-report-shows.html

Obire, O., 1988. Studies on the biodegradation potential of some micro-organisms isolated from water systems of two petroleum producing areas in Nigeria. Nigerian Journal of Botany Volume I. www.botanicalsocietyofnigeria.org/volume1.htm

Obire, O., and Putheti, R. R. 2009. Fungi in Bioremediation of Oil Polluted Environments.

Obire, O.; and Anyanwu, E. C., 2009. Impact of various concentrations of crude oil on fungal populations of soil. Int. J. Environ. Sci. Tech., 6 (2), 211-218, Spring 2009

Punekar, N. S., Vaidyanathan C. S. and Rao, A. N., Role of glutamine synthetase in citric acid fermentation by Aspergillus niger. J. Biosci., Vol 7, Numbers 3 & 4 June 1985, pp 269–287. India.

Sebiomo, A., Bankole, S. A. and Awosanya, A. O. 2010. Determination of the ability of microorganisms isolated from mechanic soil to utilise lubricating oil as carbon source. African Journal of Microbiology Research Vol. 4(21), pp. 2257-2264, 4 November, 2010. http://www.academicjournals.org/ajmr

Sood, N., Lal, B. 2009. Isolation of a novel yeast strain Candida digboiensis TERI ASN6 capable of degrading petroleum hydrocarbons in acidic conditions. Journal of Environmental Management. 2009 Apr; 90(5):1728-36. Epub 2008 Dec 25. http://www.ncbi.nlm.nih.gov/pubmed/19111380

The Ghanaian Times, Oil Spill: Cosmos May Pay Penalty to Govt. Thursday, 7th January 2010. http://www.newtimes.com.gh/story/1538

Vogt, J., 2010. BP Oil Spill: Chemical Dispersants Threaten Gulf. The Fiscal Times. May 28, 2010. http://www.thefiscaltimes.com/Articles/2010/05/28/BP-Oil-Spill-Chemical-Dispersants-Threaten-Gulf.aspx