Isolation and Characterization of Hydrocarbon Biodegrading Fungi from oil contaminated soils in Thika, Kenya

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A thesis submitted in partial fulfillment for the degree of Master of Science in Mycology in the Jomo Kenyatta University of Agriculture and Technology

2015

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature……Date……
…

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This thesis has been submitted for examination with our approval as the University supervisors.

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DEDICATION

This work is dedicated to my dear husband Robert Nesta, and to my children, Frank Osteen and Angela Sifa. Your prayers and encouragement made me strong. May the God almighty, bless you.

ACKNOWLEDGEMENTS

I thank the Almighty God for the knowledge, strength and good health which he has enabled me with since I started my studies. I am greatly indebted to Jomo Kenyatta University of Agriculture and Technology, Research production and Extension for research funds they availed to me. My sincere gratitude goes to my supervisors Prof. Viviene Matiru and Prof. Sheila Okoth for their support, patience, guidance and insightful ideas they gave me during the period of my research. I also thank Prof. Nancy L.M Budambula for supporting me in many ways.

I also express my gratitude to Mr. Benson Mbugua who helped me during soil sampling. Mr. Josephat Muthanga, for his assistance on the various experiments. I also thank Mr Rotich of Institute for Biotechnology Research, JKUAT, for his support in the laboratory. I thank Agnes Omire and all other special friends and colleges in the Botany department and other departments for their assistance.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
Ca	Calcium
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
g	Grams
GC-MS	Gas Chromatography- Mass Spectrometry
HCl	Hydrochloric acid
IBR	Institute of Biotechnology Research
JKUAT	Jomo Kenyatta University of Agriculture and Technology
K	Potassium
KCl	Potassium Chloride
M	Molar
ml	Milliliters
MEGA	Molecular Evolutionary Genetic Analysis
µL	Microlitres
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
PAHs	Polycyclic aromatic hydrocarbons
PCR	Polymerase Chain Reaction
RPE	Research production and Extension
rRNA	ribosomal Ribonucleic Acid
SDA	Sabourand dextrose agar
SDS	Sodium dodecyl Sulphate
TAE	Tris- Acetic acid Ethylene diamine tetra-acetic acid
TBE	Tris- Boric acid Ethylene diamine tetra-acetic acid
TRIS	Tris-(hydroxymethyl) - aminomethane

ABSTRACT

Spillage and extensive exploration of petroleum products results in pollution of the environment. Bioremediation of the oil contaminated sites could be achieved by fungal biodegradation; however, the specific fungi involved have not been determined in Kenya. In this study fungal isolates from Thika in central Kenya were screened for biodegradation of engine oil. There sample size was four garages that were more than 10 years. Soil was homogenously mixed for each of the twenty seven soil samples. The initial isolation from the oil contaminated soil was done using potato dextrose agar at a temperature of 30°C for seven days. The biodegradation incubation was done using Bacto Bushnell – Haas broth at 30°C for twenty one days. Colonies were observed using a light microscope at a magnification of x1000 and characterized morphologically. The 18S rRNA genome was amplified, sequenced and the sequences used for phylogenetic analysis. The size of the amplicon targeted was 700bp in approximation. The isolates were grown at varied temperatures and pH, and screened for enzymatic activities. Analysis for the biodegraded oil was done by gas chromatography- mass spectrometry (GCHRGC 400B - MSQ12 Konic-Spain). Eight fungal isolates were recovered from polluted soils namely, *Trichoderma viride, Trichoderma spirale, Neosartorya pseudofischeri, Neosartorya aureola, Aspergillus flavus, Aspergillus terreus, Penicillium griseofulvum* and *Trichoderma longibrachiatum*. Comparison of the 18S rRNA gene sequences to known fungal sequences in the Genbank database using BLAST analysis indicated similarity of more than 97%. The percentage similarity for isolates that biodegraded oil namely, *Penicillium griseofulvum* and *Aspergillus flavus* was 99% and 100%. The eight fungi were characterized morphologically and were different from each other through different parameters like colour, elevation and margin among others. Out of eight fungal isolates recovered from contaminating soil, only *Aspergillus flavus* and *Penicillium griseofulvum* biodegraded seven and seventeen oil compounds respectively. However, some compounds could not be fully biodegraded by

Aspergillus flavus namely, decane, undecane and tridecane from a concentration of 5.48 to 0.23, 18.14 to 0.13 and 14.22 to 0.11 mg/l respectively. *Penicillium griseofulvum* could not fully biodegrade 1-Ethylidene-1–Indene from a concentration of 0.29 to 0.17mg/l. The optimum growth temperature range for the eight fungi was 30^{o}C and 40^{o}C. There was no growth at 50^{o}C for all isolates except some slight growth by *Aspergillus flavus*. Optimum growth at pH 7 and pH 9 and poor growth at pH 5 was noted. The eight fungi produced amylase, protease, lipase/esterase and cellulase enzymes. This study will contribute to the database on locally available fungal diversity and their ecology. This will also increase knowledge of the fungi involved in biodegradation of oil in Kenya. Moreover Kenya's Turkana has recently discovered oil and so this could be of great help in dealing with events of oil spill so as to conserve our precious environment.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Background Information

Crude oil is the oily, flammable liquid that occurs naturally in deposits beneath the surface of the earth. Crude oil or petroleum is a viscous liquid that consist of hydrogen and carbon ions (Asep & Sanro, 2013). Engine oil is a pollutant in the environment and causes damage to our ecosystem and a health hazard to human beings (Fatuyi *et al.,* 2012). After any oil spillage, Polycyclic Aromatic Hydrocarbons (PAHs) are important contaminant retained in the environment (Kathi & Anisa, 2012). Oil pollution has contaminated soils used for agricultural lands and has not spared the aquatic and marine plants and animals in Nigeria. Ground water has also been contaminated hence polluting the crops and farm animals (Eneh, 2011) Huge increase of vehicles due to rapid increase of human population has led to presence of various kinds of informal and formal automobiles hence increased use of motor oil (Husaini *et al.,* 2008). The toxicity of oil and other petroleum products varies depending on the concentration, composition, environmental factors and biological state of the organism at the time of contamination (Cerniglia & Setherland, 2001).

Petroleum hydrocarbons can be divided into four classes: saturates, aromatics, asphaltenes (phenols, fatty acids, ketones, esters & porphyrins), and the resins (pyridines, quinolines, carbazoles, sulfoxides & amides) (Colwell *et al.,* 1977). Petroleum hydrocarbon compounds bind to soil components, and they are difficult to be removed or degraded (Barathi & Vasudevan, 2001). Hydrocarbons differ in their susceptibility to microbial attack. The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkane's althoughthis is not always the case (Perry, 1984). Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be biodegraded at all (Atlas & Bragg, 2009).

Fungi and bacteria have been used in the past for biodegradation (Snape *et al.*, 2001). The filamentous fungi possess some attributes that enable them to be good potential agents of degradation. A fungus attaches itself quickly on the substratum then digests the substratum through the secretions of extracellular enzymes (Okerentugba & Ezeronye, 2003). Fungi are capable of growing under environmental stress including, low pH, poor nutrients and low water activity. Fungal bioremediation is an attractive approach over other techniques like physical-chemical for it is simple, easy to maintain, cost effective and can be produced in mass (Achal *et al.*, 2011). Studies by Smita *et al.*, (2012) shows that, *Aspergillus, Penicillium, Fusarium, Rhizopus, Alternaria* and *Cladosporium* species have been identified as hydrocarbon biodegraders. This study aimed to isolate and identify hydrocarbon biodegrading fungi from engine oil contaminated soils in Thika, Kenya.

1.2 Literature review

1.2.1 Oil pollution

Petroleum products are extensively widespread all over the world and their intensive use is strongly connected to heavy discharge of hydrocarbons into the environment (Winkelmann *et al.*, 2009). A major concern for petroleum hydrocarbon bioremediation is the presence of heavy compounds such as polycyclic aromatic hydrocarbons (PAHs). These heavy hydrocarbon constituents are considered potential health risks due to their possible carcinogenic and mutagenic actions (Baheri & Meysami, 2002). Moreover, lubricant oil can persist for more than six years in some ecosystems, resulting in chronic problems to the biota (Burns *et al.*, 1994). Wide use of petroleum hydrocarbon in factories leads to pollution caused by production processes. Pollution occurs both in marine and aquatic environment for example harbors, but also in the terrestrial environment, mostly near factories or tank stations. Soil pollution is not considered as a big environmental problem due to the restriction of the pollution to a small local area. Local areas polluted with petroleum are numerous (Hoeppel & Hinchee, 1991), and it is important to develop techniques to efficiently clean these areas. In the terrestrial environment, the contaminating organic compounds first migrate downward in the soil due to gravity. Heavier

compounds go further spreading in the layer above the ground water, while the lighter parts continue their way downwards up to the water table. This is of interest for soil pollution, because it causes spreading of the pollution and creates the possibility of organic compound entering in the ground water used for a variety of purposes (Qin & Huang, 2009). Oil in Niger Delta is lost because of the geographical terrain of which is mostly a mangrove swamp and marsh. The soft flowing mud of the swamps and prop roots of the mangrove trees are usually the natural obstacles during oil recovery efforts in this region; through which heavy machinery cannot be moved for oil recovery efforts. Bioremediation is therefore can be a good answer to the removal of oil spilled in these areas and the best means of remediation in such ecosystems (Azaiki, 2009).

1.2.2 Remediation Process

Physical, chemical and mechanical processes are traditional methods used in remediation of contaminated areas. Physical remediation method includes incineration, brick making and skimmers etc. This method cannot biodegrade more than 10-15% of spilled oil (Thavasi *et al.,* 2011). Use of chemical surfactants as remediating agent on the other hand is not favourable due to their toxic effects on flora and fauna (Thavasi *et al.,* 2011). However, this type of treatment system requires heavy machinery and the environmental consequences of this pollutant removal may result in massive air pollution (Bhupathiraju *et al.,* 2002).

Despite decades of research, successful bioremediation of oil contaminated environment stills remains a challenge (Perfumo *et al.,* 2010). Allying high efficiency and low costs, bioremediation processes represent an extremely important way of recovering oil contaminated areas among several other remediation processes (Bhupathiraju *et al.,* 2002). Bioremediation is the conversion of chemical compounds into energy, cell mass and biological waste products using living organisms, especially microorganisms (Rahman *et al.,* 2002). Bioremediation converts contaminants to harmless end products and the process is therefore economical and efficient (Das & Mukherjee, 2007).

1.2.3 Phytoremediation

Phytoremediation' can be defined as plant enhanced decontamination of soil and water. Contaminants may be degraded, extracted from soil or contained in the plant. Plants used in phytoremediation must tolerate the pollutants at a concentration present in contaminated environments (Wenzel *et al.,* 1999). Phytoremediation research dealing with oil contaminants is focused to root zones, since more than a half of the biomass of most plants is located in roots (Adams *et al.,* 2000). Phytoremediation does not require an external energy source other than sunlight, if no artificial irrigation is used. Phytoremediation mechanism active in hydrocarbon decontamination include plant uptake and phytodegradation. Plants can take up hydrocarbons to their roots and above ground biomass (Chaineau *et al.,* 1997). This method of degradation should be used at low and moderate levels of contamination, which includes moderate hydrophobic organic compounds such as chlorinated solvents, benzene, toluene and ethylbenzene and short chain aliphatics that are readily taken up and translocated within the plants (Adams *et al.,* 2000). Plant uptake of organic chemicals such as PAHs is plant species dependent. Most of these studies show that plants enhanced removal of hydrocarbons, although removal efficiency of aged contaminants was lower than that of freshly spilled contaminants. Alfalfa plant has been known to biodegrade crude oil (Wiltse *et al.,* 1998). Annual Ryegrass and Yellow sweet clove has also been known to biodegrade aged PAHs from manufactured gas plant (Parrish *et al.,* 2004). The degradative enzymes in the plants include laccase, peroxidase and cytochrome P450 (Chroma, 2002).

1.2.4 Algae

Algae have the possibility of degrading hydrocarbons, and more specific PAHs. They use the mechanism similar to fungi (Warshawsky & Cody, 1995). Algae depend on light for them to be able to degrade PAHs. Studies done by Manoz & Guieysee (2003) showed differences in degradation rate for different algae. Polycyclic Aromatic Hydrocarbons can be degraded or accumulated in the algae as biomass. Some algae only accumulate, while other degrades nearly all PAHs (Muñoz, & Guieysee, 2003).

1.2.5 Bacteria

Bacteria are able to biodegrade petroleum compounds especially the PAHs. Degrading bacteria are present in both the water and the soil environment (Prince *et al.,* 2003). The degradation speed of hydrocarbons depends on the compound, the bacteria present and the environmental condition. Bacteria and fungi are key agents of biodegradation, with bacteria being dominant in marine ecosystems while fungi are more dominant in freshwater and terrestrial environment although this may vary from one region to another (Colwell & Walker, 1977).

1.2.6 Fungi

Fungi are known to biodegrade hydrocarbons. Mechanisms used by fungi are different from that of bacteria for hydrocarbons degradation. Fungi are therefore able to degrade the hydrocarbon compound left by the normally faster degrading bacteria. This can be useful for the five-ring PAHs, which are only poorly degraded by bacteria. Fungi secrete extracellular oxidizing enzymes for degradation of lignin (Field & Jong, 1992). These enzymes are able to make reactive peroxide from oxygen (Barr & Aust, 1994). Lignin is a complex random molecule contained in most aromatic groups. Fungi biodegrade hydrocarbons by penetrating the mycelia in the oil and increase the surface area available for degradation by other microbes.

Fungal species belonging to the genus *Tichoderma* are worldwide in occurrence and easily isolated from soil, decaying wood, and other forms of plant organic matter. They are classified as imperfect fungi, since most of them have no known sexual stage. They grow rapidly in culture and produce numerous spores (conidia). Many species produce large quantities of thick-walled spores (chlamydospores) in submerged mycelium (Gams, 1998). Most *Trichoderma* species especially *Trichoderma harzianum* is used as biocontrol agent of plant diseases and was first recognized in the early 1930s (Horie *et al.,* 2003). Obire *et al.* (2008) found *Aspergillus* species to biodegrade oil.

The fungal genus *Neosartorya* belongs to phylum Ascomycota which has a worldwide distribution in the soil. *Neosartorya pseudofischeri* is the sexual stage of *Aspergillus thermomutatus* (Jarv *et al.*, 2004). *Neosartorya* is likely to be present along with related *Aspergillus thermomutatus* if growth has been there for long and the nutrients of the substrate are conducive for the conversion to sexual phase. *Neosartorya pseudofischeri* causes illnesses that include pulmonary infection, endocarditis, and osteomylelitis related to those of *Aspergillus* anamorph and have rarely been studied. Spores have somewhat distinctive morphology but would most probably be called "ascospores" on spore trap samples (Jarv *et al.*, 2004).

The *Aspergillus* genus has always been present in the human environment. Raper & Fennel, 1965 reported that Micheli was the first to distinguish stalks and spore heads. In the middle of the 19th century that these fungi began to be recognized as active agents in decay processes, as causes of human and animal disease and as fermenting agents capable of producing valuable metabolic products (Raper & Fennel, 1965). *A. flavus* is second from *A. fumigatus* as the cause of human invasive Aspergillosis. In addition, it is the main *Aspergillus* species infecting insects and it is also able to cause diseases in economically important crops, such as maize and peanuts, and to produce potent mycotoxins (Campbell, 1994). *Aspergillus flavus* has a worldwide distribution. This probably results from the production of numerous airborne conidia, which are easily dispersed by air movements and possibly by insects. Atmosphere composition has a great impact on mould growth, with humidity being the most important variable (Gibson *et al.*, 1994). *Aspergillus flavus* appears to spend most of its life growing as a saprophyte in the soil, where it plays an important role as a nutrient recycler, supported by plant and animal debris (Scheidegger & Payne, 2003). The fungus overwinters either as mycelium or as resistant structures known as sclerotia. The sclerotia either germinate to produce additional hyphae or they produce conidia (asexual spores), which can be further dispersed in the soil and air (Bhatnagar *et al.*, 2000).

Aspergillus terreus, also known as *Aspergillus terrestrius*, is a fungus found worldwide in the soil. This saprotrophic fungus is prevalent in warmer climates such as tropical and subtropical regions (Shimada *et al.,* 2002). Aside from being located in the soil, *A. terreus* has also been found in habitats such as decomposing vegetation and dust (Arabatzis & Velegaki, 2013). *Aspergillus terreus* is commonly used in industry to produce important organic acids, such as itaconic acid as well as enzymes, like xylanase. It was also the initial source for the drug mevinolin (lovastatin), a drug for lowering serum cholesterol (Arabatzis & Velegaki, 2013). *Aspergillus terreus* can cause opportunistic infection in people with deficient immune systems. It is relatively resistant to amphotericin B, a common antifungal drug. *Aspergillus terreus* also produces aspterric acid and 6-hydroxymellein, inhibitors of pollen development in *Arabidopsis thaliana* (Shimada *et al.,* 2002).

Penicillium is a genus in the phylum ascomycota and contains over 300 species. *Penicillium* species are of major importance in the natural environment as well as food and drug production. Some members of the genus produce penicillin, a molecule that is used as an antibiotic, which kills or stops the growth of certain kinds of bacteria inside the body. Other species are used in cheese making according to the dictionary of the Fungi (10th edition, 2008).

Fungi are notably aerobic and can also grow under environmentally stressed conditions such as low pH and poor nutrient status, where bacterial growth might be limited. Finally, fungi are easy to transport, genetically engineered, and produce in large quantities. In petroleum-producing regions of Nigeria, Obire, (1988) found several species for oil-degrading aquatic fungi in the genera *Candida, Rhodotorula, Saccharomyces* and *Sporobolomyces* (yeasts) and, among filamentous fungi, *Aspergillus niger, Aspergillus flavus, Blastomyces sp, Botryodiplodia theobromae, Fusarium sp, Nigrospora sp, Penicillium griseofulvum, Penicillium glabrum, Pleurofragmium sp,* and *Trichoderma harzianum.* More examples of fungal isolates that have been found to biodegrade engine oil are shown in Table 1.1

Table 1.1: Examples of oil degrading fungal genera isolated in the past

Reported oil degrading fungal genera isolated from contaminated soils	**Reference**
Acremonium	Llanos & Kjoller, 1976
Aspergillus	Bartha & Atlas, 1977; Obire *et al.,* 2008
Aureobasidium	Bartha & Atlas, 1977
Candida	Bartha & Atlas, 1977; Obire *et al.,* 2008
Cephalosporium	Bartha & Atlas, 1977; *Obire et al*., 2008
Cladosporium	Walker *et al.,* 1973; Bartha & Atlas, 1977; Obire *et al.,* 2008)
Cunninghamella	Bartha & Atlas, 1977
Fusarium (	Llanos & Kjoller, 1976; Obire *et al.,* 2008
Geotrichum	Obire *et al.,* 2008
Giiocladium	Llanos & Kjoller, 1976
Graphium	Llanos & Kjoller, 1976
Hansenula	Bartha & Atlas, 1977
Mortierella	Llanos & Kjoller, 1976
Mucor	Obire *et al.,* 2008
Paecilomyces	Llanos & Kjoller, 1976
Penicillium	Llanos & Kjoller, 1976; Bartha & Atlas, 1977; Obire *et al.,* 2008
Rhodosporidium	Ahearn & Meyers, 1976; Bartha & Atlas, 1977
Rhodotorula	Bartha & Atlas, 1977; Obire *et al.,* 2008
Saccharomyces	Bartha & Atlas, 1977
Sphaeropsidale	Llanos & Kjoller, 1976
Torulopsis	Bartha & Atlas, 1977
Trichoderma	Llanos & Kjoller, 1976; Obire *et al.,* 2008
Trichosporon	Ahearn & Meyers, 1976; Bartha & Atlas, 1977

1.2.7 Some environmental factors affecting biodegradation of hydrocarbons

1.2.7.1 Temperature

The most important factor is temperature, because the biological enzymes involved in the degradation pathway have an optimum and will not have the same metabolic

turnover for every temperature (Iranzo *et al.,* 2001). Temperature influences petroleum biodegradation by its effect on the physical nature and chemical composition of the oil, rate of hydrocarbon metabolism by microorganisms, and composition of the microbial community (Venosa & Zhu, 2003). Hydrocarbon biodegradation can occur over a wide range of temperatures and the rate of biodegradation generally decreases with decreasing temperature. The highest degradation rates generally occur in the range of 30 to 40°C in soil environments, 20 to 30°C in some freshwater environments, and 15 to 20°C in marine environments. The effect of temperature is also complicated by other factors such as the composition of the microbial population (Zhu *et al.,* 2001).

The 18S ribosomal RNA (abbreviated 18S rRNA) is a part of the ribosomal RNA. The S in 18S represents Svedberg units. The 18S rRNA is a component of the small eukaryotic ribosomal subunit (40S) (Gardes & Bruns, 1993). The 18S rRNA is the structural RNA for the small component of eukaryotic cytoplasmic ribosomes, and thus one of the basic components of all eukaryotic cells. It is the eukaryotic nuclear homologue of 16S ribosomal RNA in Prokaryotes and mitochondria (White *et al.,* 1993). The genes coding for 18S rRNA are referred to as 18S rDNA. Sequence data from these genes is widely used in molecular analysis to reconstruct the evolutionary history of organisms, especially in vertebrates, as its slow evolutionary rate makes it suitable to reconstruct ancient divergences (Parrish *et al.,* 2004).

The small subunit (SSU) 18S rRNA gene is one of the most frequently used genes in phylogenetic studies and an important marker for random target polymerase chain reaction (PCR) in environmental biodiversity screening. In general, rRNA gene sequences are easy to access due to highly conserved flanking regions allowing for the use of universal primers (Perfumo *et al.,* 2010). Their repetitive arrangement within the genome provides excessive amounts of template DNA for PCR, even in the smallest organisms. The 18S gene is part of the ribosomal functional core and is exposed to similar selective forces in all living beings (White *et al.,* 1990).

The Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) are highly variable sequences of great importance in distinguishing fungal species by PCR analysis. The ITS region is now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other genic regions of rDNA (SSU and LSU), variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions (Gardes & Bruns, 1993).

1.2.7.2 pH

Organic soils in wetlands are often acidic, while mineral soils have more neutral and alkaline conditions. Most heterotrophic bacteria and fungi favor a neutral pH, with fungi being more tolerant of acidic conditions (Margesin & Schinner, 2001). Studies have shown that degradation of oil increases with increasing pH, and that optimum degradation occurs under slightly alkaline conditions (Zhu *et al.*, 2001).

1.2.7.3 Salinity

Changes in salinity may affect oil biodegradation through alteration of the microbial population. Many freshwater organisms can survive for long periods in seawater although few can reproduce (Iranzo *et al.*, 2001). In contrast, most marine species have an optimum salinity range of 2.5 to 3.5% and grow poorly or not at all at salinity lower than 1.5 to 2% (Venosa & Zhu, 2003).

1.2.8 Importance of fungal enzymes in biotechnology

Many enzymes produced by fungi have relevant biotechnological applications in several industrial areas. Filamentous fungi are the preferred source of industrial enzymes because of their excellent capacity for extracellular protein production (Schaffner & Toledo, 1991). Among a large number of non-pathogenic microorganisms capable of producing useful enzymes, filamentous fungi are particularly interesting due to their easy cultivation, and high production of extracellular enzymes of large industrial potential. These enzymes are applied in the industrialization of detergents, starch, drinks, food, textile, animal feed, baking, pulp

and paper, leather, chemical and biomedical products. The use of starch degrading enzymes was the first large-scale application of microbial enzymes in the food industry (Bennett, 1998). Amylases have applications in food, detergents, drinks, animal feed and baking (Pandey *et al.,* 2000). Extracellular enzymes target macromolecules such as carbohydrates (cellulases), lignin (oxidases), organic phosphates (phosphatases), amino sugar polymers (chitinases) and proteins (proteases) and break them down into soluble sugars that are subsequently transported into cells to support heterotrophic metabolism (Sinsabaugh, 1994).

1.3 Statement of the Problem

Oil contamination has serious impact on the surrounding environment. Large amount of used engine oil is liberated into the soil through motor cars, motor bikes, farmland machines and oil changing operations which are mostly conducted at garages and at fuel stations. The oil enters in to the soil when left openly without taking good measures of disposal. Spent engine oil, when present in the soil creates an unsatisfactory condition for life in the soil like plants, useful microorganisms i.e. decomposers. Oil in the soil causes poor aeration in the immobilization of nutrients and lowering of pH. Farmers in the oil exploration areas in Kenya are likely to experience tremendous difficulties in restoring the fertility of oil devastated farmlands, due to lack of knowledge on appropriate remediation procedures. No studies in Kenya have been done on the potential use of fungal biodegradation on hydrocarbons and the useful enzymes they have.

1.4 Justification of the Study

Rapid industrialization can only move hand in hand with the efficient and optimum feasibility of transport, which results into increased use of garages. With the increase in vehicle use, Kenya has witnessed a huge increase in number of garages and also the service stations where significant amount of used motor oil is erroneously discharged in to the environment. The used motor oil during manual oil changing operation is not recycled but spilled and dumped at station sites, thereby polluting both soil and water.

Kenya's Lake Naivasha has suffered from pollution and this has led to the death of aquatic life that included fish a source of food to the communities around. Nairobi River has not been spared either from oil pollution (Okoth & Otieno, 2001). Sustainable use of fungi in hydrocarbon biodegradation in Kenya remains to be one of the better options in dealing with oil contaminated soils. This is because fungal bioremediation is typically less expensive than the equivalent physical-chemical methods. It also requires little energy input and preserves the soil structure. This study will contribute to the database on locally available fungal diversity of oil contaminated environment and their ecology. This will also increase knowledge of the fungi involved in biodegradation of oil in Kenya. Moreover Kenya's Turkana has recently discovered oil and so this could be of great help in dealing with events of oil spill so as to conserve our precious environment.

1.5 Hypotheses

1. Oil contaminated soils in Thika town do not harbour fungi
2. Oil contaminated soils in Thika town do not contain hydrocarbon biodegrading fungi.

1.6 Objectives

1.6.1 General Objective

To isolate, characterize and identify fungi capable of biodegrading hydrocarbons in Thika town.

1.6.2 Specific Objectives

The specific objectives of this study were:

1. To isolate, characterize and identify fungal isolates and determine their ability to biodegrade oil from oil contaminated soils in Thika town.
2. To carry out molecular characterization of the fungal isolates using 18S rRNA based analysis.
3. To screen the isolates for useful extracellular enzymes.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Study site

Samples of the oil contaminated soils were obtained from four informal automobiles garages in Thika, an industrial town in November 2012. Thika town has a total area of approximate 220.2 km^2. The town lies between longitude 37 ^{o} 04' 09" E, latitude 1 ^{o} 01' 59" S and elevation above sea level of 1506m = 4940 feet in Kiambu County, Central Kenya (Fig 2.1).

2.2 Sample size

A pilot study identified four informal autogarages that were more than ten years old. The identified garages were randomly sampled in the study site. The sample size was determined using the following formula $n = \frac{N}{1+N(e^2)}$ (Israel, 2009).

Where n= sample size

N= total number of garages in Thika town that are over 10 years old

e= desire margin of error of 10% between the sample mean and the population mean at α= 0.05.

$$n = \frac{4}{1+4\,(0.05^2)}$$

$$= 4$$

2.3 Soil sampling

A total of twenty-seven soil samples were collected from where the engine oil was frequently spilled. The samples were collected randomly from different sites 15cm below the soil surface. The soil samples weighing one kilogram each were taken consecutively after tilling with a sterile scoop and transferred into sterile polythene bags and stored in a refrigerator of 4°C before analysis.

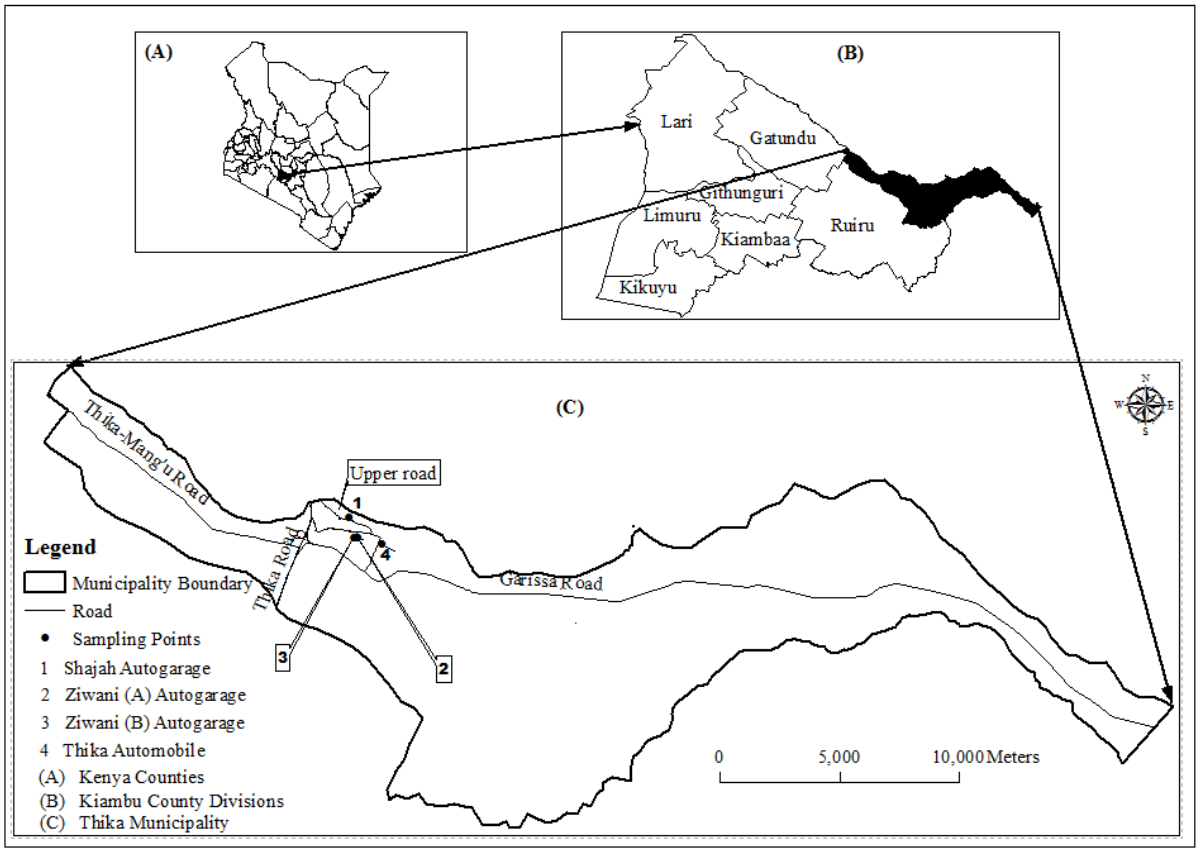


Figure 2.1: Map showing location of sampling points in Thika Town.

2.4 Isolation of fungi from oil contaminated soils

Collected soil samples were homogeneously mixed and carefully sorted to remove stones and other unwanted soil debris using a 2.5 mm sieve. The PDA media was autoclaved at 121^{o}C for 15, min allowed to cool and 20ml dispensed aseptically on the sterile disposable petri dishes. One gram of each sorted soil sample was homogenously mixed with 1 drop of Tween 80 to enhance biodegradation and was later sprinkled onto the Potato Dextrose Agar (PDA) media and incubated for 7 days at 30^{o}C. Ampliclox 25mg/l was added to the media after autoclaving to prevent contamination by bacteria. The pure colonies were selected based on morphological characteristics (Aneja, 2005).

2.5 Morphological characterization

To purify the fungal isolates, the cultures were carefully and aseptically sub cultured on Potato Dextrose Agar (PDA) and stored on PDA slants for further analysis at -

4°C. The fungal isolates were characterized on the basis of cultural characteristics and morphological characteristics including spore type, mycelia and other fruiting bodies in a lactophenol cotton blue wet mount by compound microscope at magnification of ×1000. Observed characteristics were recorded and compared with the established identification key by Barnett and Hunter (1972).

2.6 Glass slide culture technique

Slide cultures are of great value in determining details of fungal growth. The Potato Dextrose Agar was prepared as recommended by the manufacturer and after cooling; it was cut into small blocks and placed on clean slides. A sterile needle was used to pick a small amount of sub- cultured fungi and inoculated at the edge of the blocks and placed cover slips at the top. Each slide held one block of the fungi. Moistened sterile blotting papers were placed at the bottom of the plates and incubated at room temperature 30^{o}C. Incubation took seven days of which was followed by characterization. By doing this, there is no need to remove a portion of the fungus from a culture plate and transfer it to the slide. So there is less chance for the features that are key to identification, notably the spore-bearing structures, to be damaged (Riddell, 1950).

2.7 Molecular characterization

2.7.1 DNA Extraction

Total genomic DNA of the isolates was extracted from cells in triplicate using SDS as a lysis buffer. A 10% SDS was used, made of 32.5 ml ddH_2O, 1MTris (pH 8.0), 5M NaCl and 0.5M EDTA (pH8.0). Seven day old cultures were scrapped aseptically into sterile eppendorf tubes containing 0.1g sterile sieved sand. Scrapping was done using sterile surgical blade taking care not to pick the media. The cultures were crushed separately in 200µl SDS buffer using sterile micro pestles in an eppendorf tube as initial crushing and later 200µl SDS was added for complete lyses of the cells. Incubation of the cells was at 65^{o}C for one hour in a water bath and then centrifuging for 10 minutes at 10rpm. The supernatant was transferred to sterile eppendorf tubes. Phenol chloroform was added in equal volume as the supernatant and gentle shaking done to prevent breaking of the DNA strands.

Phenol chloroform was used to remove the proteins by centrifuging at 10rpm for 10 minutes to separate the DNA from debris. In the supernatant, same volume of chloroform isoamyl as the supernatant was added to wash the fatty tissues and phenol by gentle shaking. Centrifuging was at 10rpm for 10 minutes and same volume of isoamyl was added in the supernatant to completely remove fatty tissues and phenol from the DNA. The aqueous phase was transferred to a new eppendorf tube and DNA stabilized by addition of 1.0 volume of 3 M NaCl and precipitated with equal volume of absolute ethanol. Precipitation was done for one hour or overnight at -2^{o}C. The precipitates were centrifuged for 10 minutes at 1300rpm and the supernatant discarded. The pellet that contained the genomic DNA was rinsed with 70 % ethanol to remove traces of salts. The DNA was dried in an incubator at 40^{o}C for fifteen minutes to completely remove the ethanol and then dissolved in 40µl TE buffer pH8. The presence of DNA was checked using 1% agarose in 1x TAE buffer and visualized under ultraviolet by staining with ethidium bromide. The DNA was stored at - 20^{o}C for subsequent use (Sambrook *et al.,* 1989).

2.7.2 Amplification of 18S rRNA gene

Total DNA from each isolate was used as a template for amplification of the 18S rRNA genes. The 18S rRNA gene was amplified using ITS1 (forward primer) TCCGTAGGTGAACCTGCGG and ITS4 as (reverse primer) TCCTCCGCTTATTGATATGC. The size of the amplicon targeted was 700bp. Amplification was performed using (Peqlab primus 96 PCR machine). Amplification was carried out in a 20µl mixture containing 7µl PCR water, 10µl master mix, 1µl forward primer, 1µl reverse primer and 1µl template DNA. The control contained all the above except the DNA template. Reaction mixtures were subjected to the following temperature cycling profiles. Initial denaturation (94^{o}C for 5 min), 40 cycles of denaturation (94^{o}C for 1.3 min) annealing (50^{o}C for 1.5 min), Extension (72^{o}C for 2min) and final elongation (72^{o}C for 10min) then -4°C soak (White *et al.,* 1990).

2.7.3 Purification of PCR products

The PCR products were purified using the QIAquick PCR purification Kit protocol (2010) according to manufacturer's instructions.

2.7.4 Phylogenetic data analysis

Sequencing of purified PCR products was done at Macrogen Netherlands. The 18S rRNA gene sequences was compared to sequences in the public database using Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) website (*http://www.ncbi.nih.gov*) in order to determine similarity to sequences in the Gen Bank database (Shayne *et al*., 2003). Phylogenetic analysis was performed using neighbor-joining method and maximum composite likelihood (MCL) methods (Tamura *et al.*, 2004) of MEGA version 6.0 software, with 1,000 bootstrap replicates.

2.8 Biodegradation assay for used and unsed engine oil

Bacto Bushnell – Haas broth containing $MgSO_4$ (0.2 g L^{-1}), $CaCl_2$ (0.02 g L^{-1}), KH_2PO_4 (1 g L^{-1}), $FeCl_2$ (0.05 g L^{-1}) and NH_4NO_3 (1 g L^{-1}) was used. Tween 80 (0.1%) redox reagent (2%) and (1% v/v) of both spent and unspent engine oil (Castrol RX Super plus, SAE 15W140) were all incorporated into the broth in 250ml conical flask. Using a cork borer of 10mm of each pure isolate was inoculated into the broth. A control flask with oil and without inocula was also maintained. Eight isolates to be assayed were replicated three times and incubated in a rotary shaker (100 rpm, 30°C) for 21 days (Das & Mukherjee, 2007). After 21 days, the broth whose colour had changed from deep blue to colorless presumably due to oxidized hydrocarbons products was centrifuged at 5000rpm for 10 minutes. Pellets were discarded and the supernatant retained.

Hexane was added having equal volume as supernatant and mixture shaken vigorously for 5 minutes and allowed to stand for 10 minutes. The top layer of hexane with the engine oil and lower aqueous layer were collected in separate clean beakers. Three grams of anhydrous sodium sulphate was used to remove the remaining moisture. Hexane was evaporated in a rotary vacuum evaporator (RE 100,

England) remaining with the biodegraded oil. The degraded oil was analyzed for identification of compounds in the oil by gas chromatography- mass spectrometry (GCHRGC 400B - MSQ12 Konic-Spain).

2.9 Sample preparation for GC-MS analysis

After biodegradation assay, 20µl of the various biodegraded engine oils were diluted using 30µl of dichloromethane and spiked with 50 µl of 1mg/ml of benzophenone, internal standard prepared in dichloromethane. Samples were kept in a refrigerator at 4°C while waiting for analysis of the compounds in the oil by GC-MS (GC HRGC 400B - MSQ12 Konic -Spain). Compound identification was done using AMDIS v 32 software.

2.10 Analysis of oil compounds in the engine oil using GC-MS

The analyses of oil compounds were performed using gas chromatograph a (GC HRGC 4000B Konic-Spain) coupled to a Mass Detector (MSQ12 Konic-Spain). Each sample measuring 1µl (prepared as above) was injected into the split less mode in a TECHNO KROMA TRB5-Spain capillary column measuring (30m × 0.25mm i.d × 0.1µm film thickness). The injection port temperature was maintained at 200^{o}C, while the oven temperature was programmed to rise from 60^{o}C to 180^{o}C at a rate of 4^{o}C/min and allowed to rise again to 250^{o}C at a rate of 6^{o}C /min with a solvent delay time of 150 seconds. Helium was used as the carrier gas at flow rate of 1 ml/min. Mass spectra were recorded in the Electron Ionization mode (MSQ12 Konic-Spain) at 70 electron volts scanning the 35-450 m/z range with a scan time of 900mS and a dwell time of 2163µS, the ion source and transfer line temperature were maintained at 200^{o}C and 250^{o}C respectively.

2.11 Physiological characterization

2.11.1 Fungal mycelial growth at different temperature

A 10mm mycelia growth plug was inoculated on PDA media and incubated at 30°C, 40°C and 50°C separately for 7 days in triplicates. The radial growth in mm for the colonies was measured and recorded. The PDA media was prepared separately according to manufacturer's instructions in conical flasks. Experiments were done in

duplicates. Positive isolates growths were detected by measuring the diameter of the colony in millimeters and recorded (Cappuccino & Sherman, 2002).

2.11.2 Fungal mycelial growth at different pH

The pH of each set of experiments was adjusted to 5.0, 7.0 and 9 with a pH meter using HCl and NaOH. The media was autoclaved, cooled and dispensed in about 20ml quantities in petri dishes. The petri dishes containing a 10mm mycelia growth disc were incubated at 30°C for a period of 7 days and the radial growth measured in millimeters and recorded. Two uninoculated plates were used as controls (Cappuccino & Sherman, 2002).

2.11.3 Screening isolates for enzymatic activity

2.11.3.1 Determination of amylolytic activity

Starch hydrolysis was done using the method of Castro (1993). Starch is a high molecular weight polymer and is hydrolyzed by the enzyme amylase. Starch agar was used to determine the hydrolytic activities of the exo-enzyme. The detection of the hydrolytic activity following growth period was made by performing the starch test to determine the presence or absence of starch in the medium. Starch in the presence of iodine imparts a blue black colour to the medium, indicating the absence of the enzyme. When starch has been hydrolyzed, a clear zone of hydrolysis surrounds the colonies hence a positive test.

2.11.3.2 Determination of the proteolytic activity

To detect protease activity, isolates were cultured on PDA media containing the following in grams per litre nutrient broth 8.0, glucose 1.0 and agar 18.0 and then pH adjusted to 8.0. PDA media was used for the protease activity. The media was supplemented with 1% of skimmed milk as sole carbon source. The isolates were inoculated and incubated at 30°C for 7 days and protease production by isolates was noted by the presence of clear halos around colonies. (Cappuccino & Sherman, 2002).

2.11.3.3 Determination of the lipolytic/esterase activity

To determine lipase/esterases activity, the isolates were cultured on basal media containing 1% KH_2PO_4, 0.01% $MgSO_4.7H_2O$, 0.005% $CaCl_2.2H_2O$, 1% NaCl and 1% Na_2CO_2 supplemented with 1% olive oil (domestic grade) as the sole carbon source. The medium was then inoculated by spotting three isolates per plate and then incubated for 7 days at 30^{o}C. The media was observed for zones of precipitation of calcium crystals around each isolate. Lipase/ esterase production was indicated by the precipitation of calcium crystals around the colonies. (Cappuccino & Sherman, 2002).

2.11.3.4 Determination of cellulolytic activity

Cellulase production was determined by using the media containing 7.0g KH_2PO_4, 2.0g K_2HPO_4, 0.1g $MgSO_4.7H_20$, 1.0g $(NH_4)2SO_4$, 0.6g yeast extract,10g microcrystalline cellulose and 15g agar per liter. The plates were inoculated and incubated at 30 °C for 7 days. For best viewing of the clear halo, the plates were stored at 50°C for one night after 7 days of incubation (Cappuccino & Sherman, 2002).

2.12 Statistical Analysis

Different samples from the garages represented the treatments. There were three replications for every treatment. Results were compared using one-way Analysis of Variance (ANOVA). This was done to establish if differences ($p<0.05$) were significant between individual treatments. The analysis was done using SPSS version 17.

CHAPTER THREE

3.0 RESULTS

3.1 Cultural characteristics of fungi from oil contaminated soils

A total of eight fungal isolates were isolated from oil contaminated soils using Potato Dextose Agar. Characterization was based on classical microscopic and macroscopic techniques of color, margin and elevation of the pure colonies. The isolates were able to grow within 4-7 days of incubation at 30 °C. The isolates exhibited different colony characteristics. Three isolates had curled margin, two had entire margin while the rest were undulate and filamentous. The isolates had raised fluffy elevation. The reverse colour was mostly creamish while the obverse varied for each isolate (Table 3.1 & Plate 3.1-3.5).

Table 3.1: Cultural characteristics of fungi isolated from oil contaminated soils in Thika.

ISOLATE CODE	ISOLATE IDENTITY	COLOUR (TOP)	COLOUR (BOTTOM)	MARGIN	ELEVATION
1	*Trichoderma viride*	Cream white	Cream	Curled	Raised fluffy
4	*Trichoderma spirale*	Grey brown	Dark brown	Entire	Raised fluffy
5	*Neosartorya pseudofischeri*	Granular white	Cream	Filamentous	Raised fluffy
12	*Neosartorya aureola*	White	Cream yellow	Entire	Medium fluffy
13	*Aspergillus flavus*	Green	Cream	Curled	Raised
14	*Aspergillus terreus*	Pinkish brown	Cream green	Curled	Raised
18	*Penicillium griseofulvum*	Dark green	Cream green	Entire	Raised fluffy
19	*Trichoderma longibrachiatum*	Cream white	Cream	Undulate	Raised fluffy

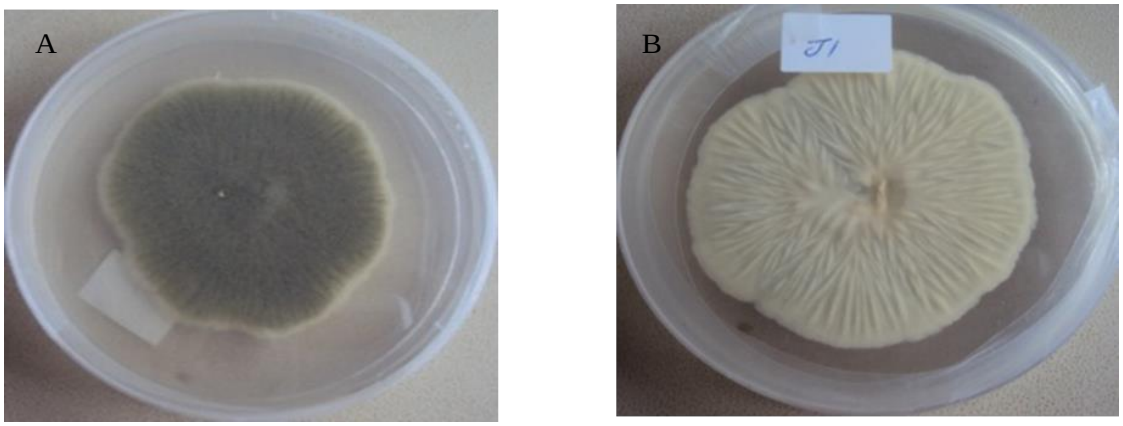


Plate 3.1: Culture plate showing A: Top of *Aspergillus flavus* on PDA media, B: Bottom of *Aspergillus flavus* on PDA media.

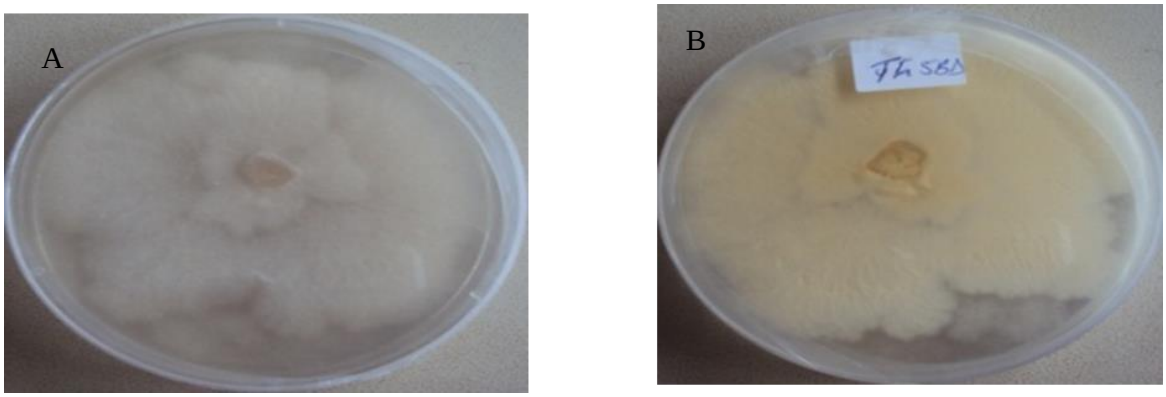


Plate 3.2: Culture plates showing A: Top of *Neosartorya aureola* on PDA media, B: Bottom of *Neosartorya aureola* on PDA media.

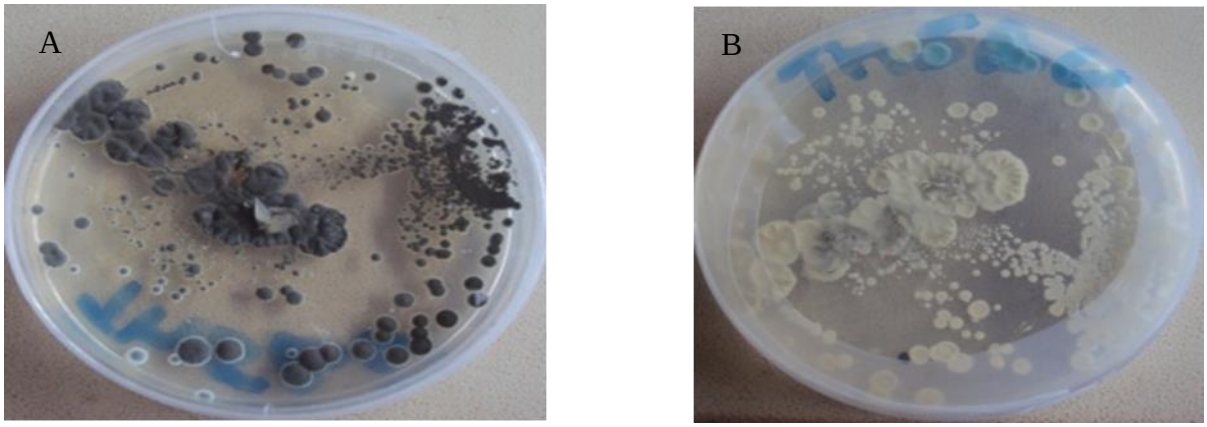


Plate 3.3: Culture plate showing A: Top of *Penicillium griseofulvum* on PDA media, B: Bottom of *Penicillium griseofulvum* on PDA media.

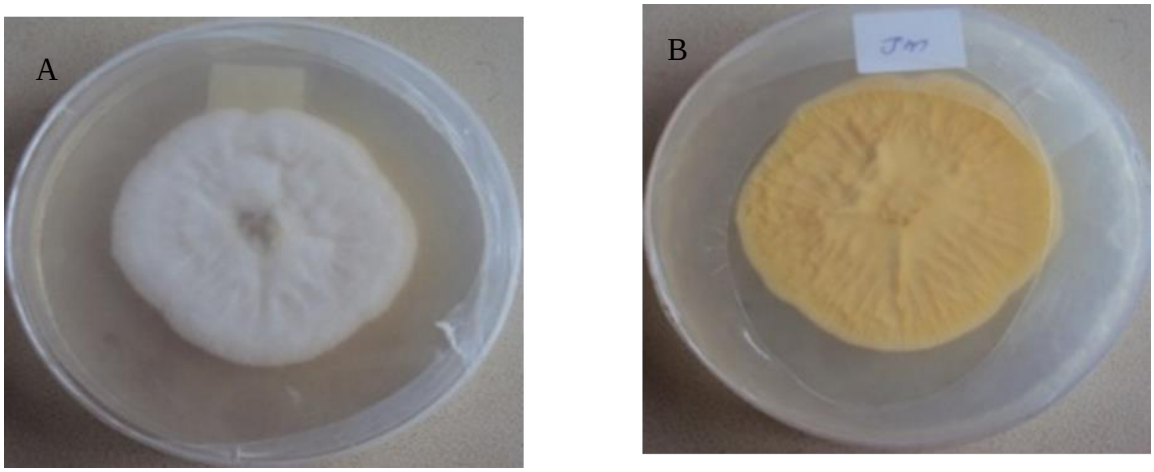


Plate 3.4: Culture plate showing A: Top of *Trichoderma viride* on PDA media, B: Bottom of *Trichoderma viride* on PDA media.

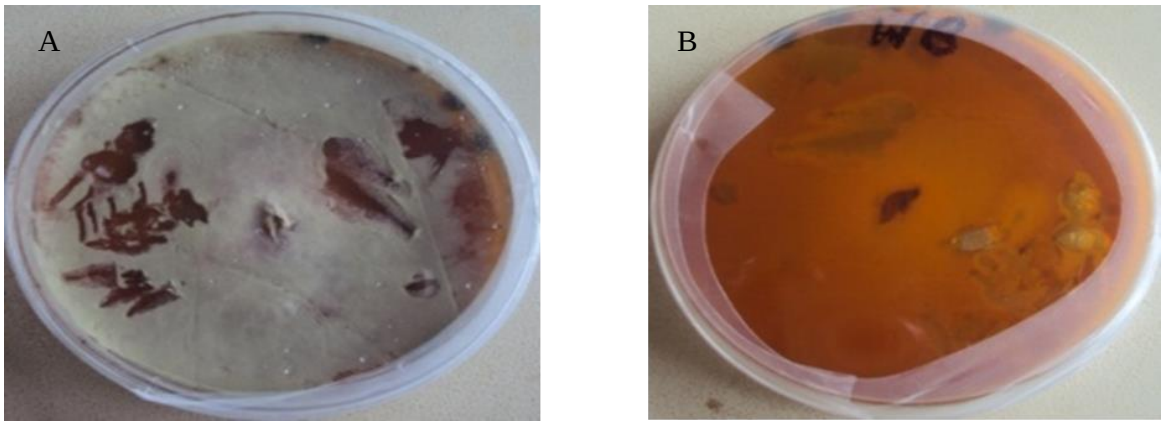


Plate 3.5: Culture plate showing A: Top of *Trichoderma spirale* on PDA media, B: Bottom of *Trichoderma spirale* on PDA media.

3.2 Microscopic Characterization of fungal isolates

Different characteristics for the isolates were noted when observed under a compound microscope at a magnification of x 1000. *Penicillium griseofulvum* had long conidiophores, long branching phialides and conidia spreading (Plate 3.6) *Aspergillus flavus* had double walled vesicles with conidia spreading from the strigmata, vesicles being flask shaped (Plate 3.7). *Aspergillus terreus* had globose vesicles after staining with Lactophenol cotton blue and had biseriate conidial head.

The conidiophores were smooth and hyaline (Plate 3.8). *Neosartorya aureola* had its conidial head forming from the conidiophore (Plate 3.9) *Trichoderma viride* had branched septated mycelia of which formed chlamydospores within the mycelia (Plate 3.10).

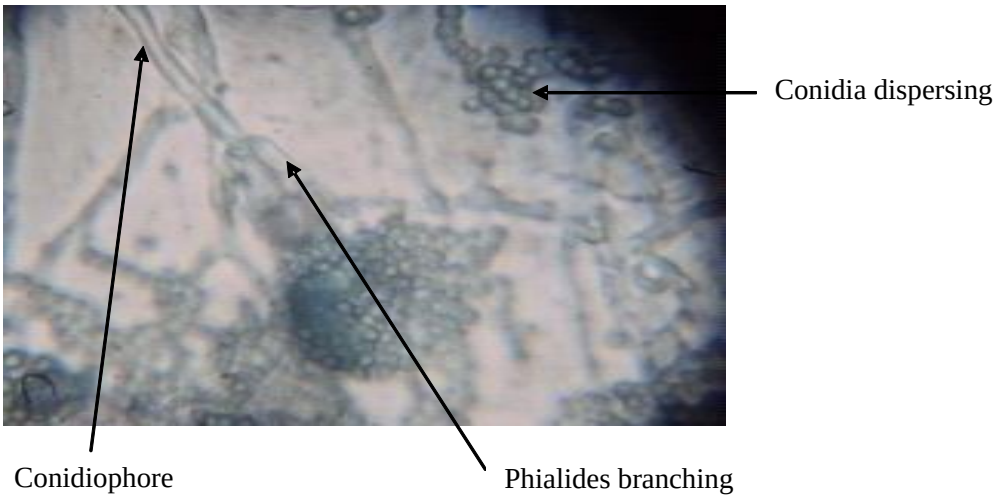


Plate 3.6: *Penicillium griseofulvum* (X 1000 magnification).

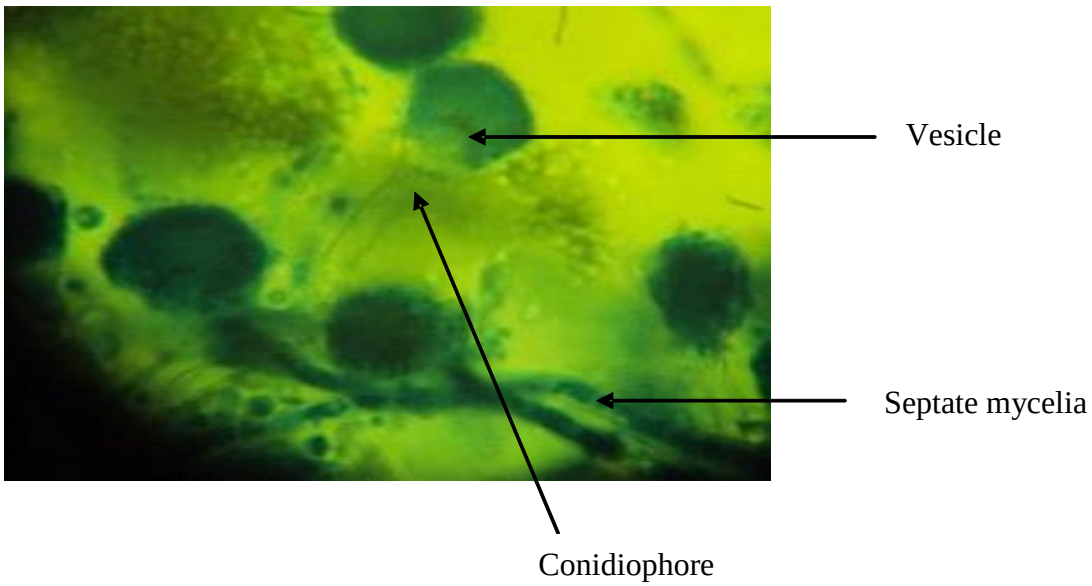


Plate 3.7: Conidiophores bearing conidium of *Aspergillus terreus* stained with cotton blue (X 1000 magnification).

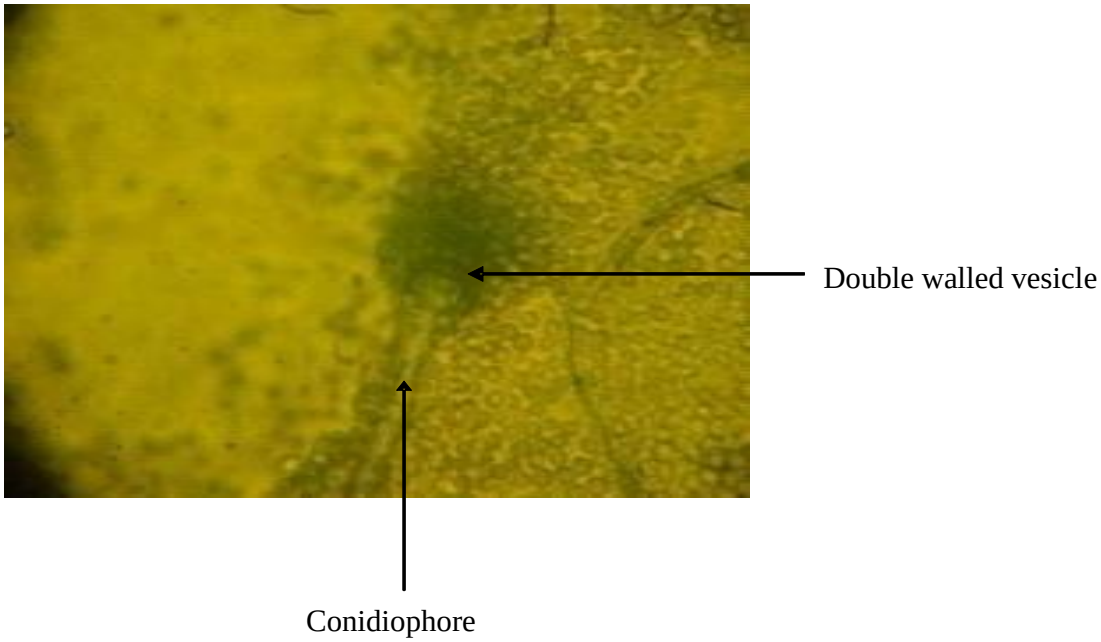


Plate 3.8: Conidiophore and conidia of *Aspergillus flavus* stained with cotton blue (X 1000 magnification).

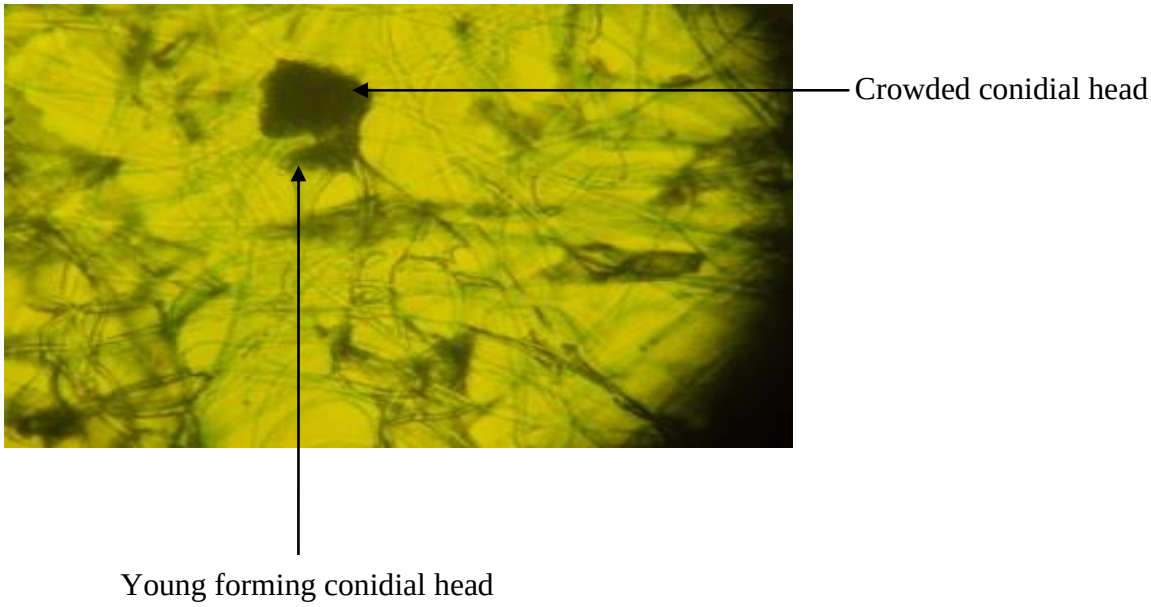


Plate 3.9: Conidiophore and mycelia of *Neosartorya aureola* (X 1000 magnification).

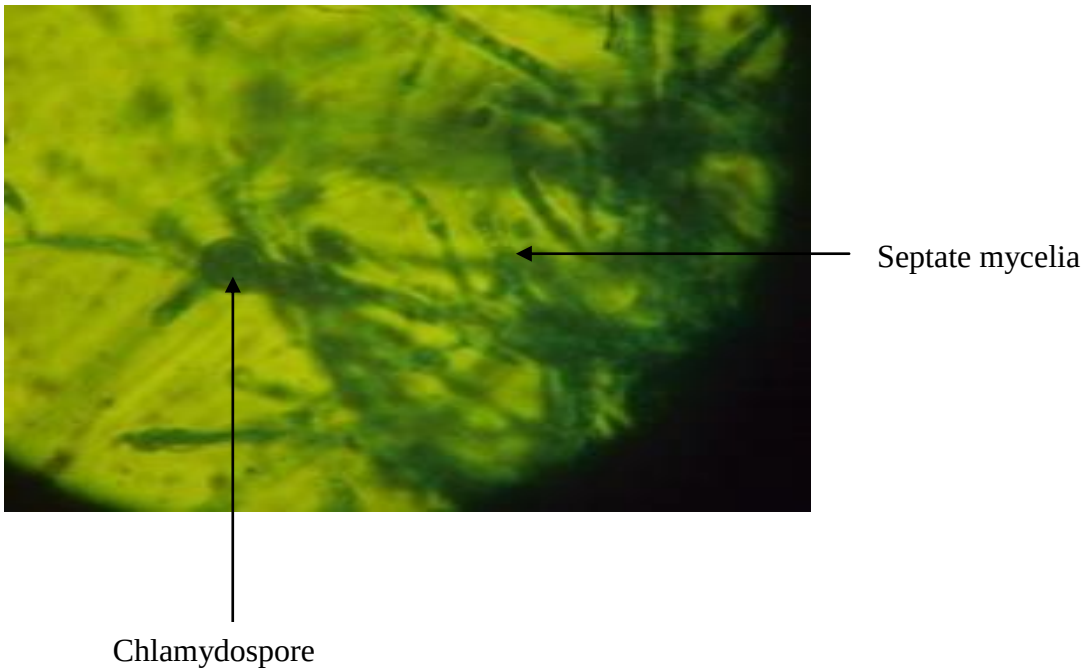


Plate 3.10: *Trichoderma viride* showing chlamydospore forming from the mycelia (X 1000 magnification).

3.3 Molecular characterization of the fungal isolates

3.3.1 PCR amplification of the fungal isolates

PCR amplification of the fungal isolates 18S rRNA using ITS 1 and ITS 4 primers yielded a product of approximately 700bp as shown in Plate 2.

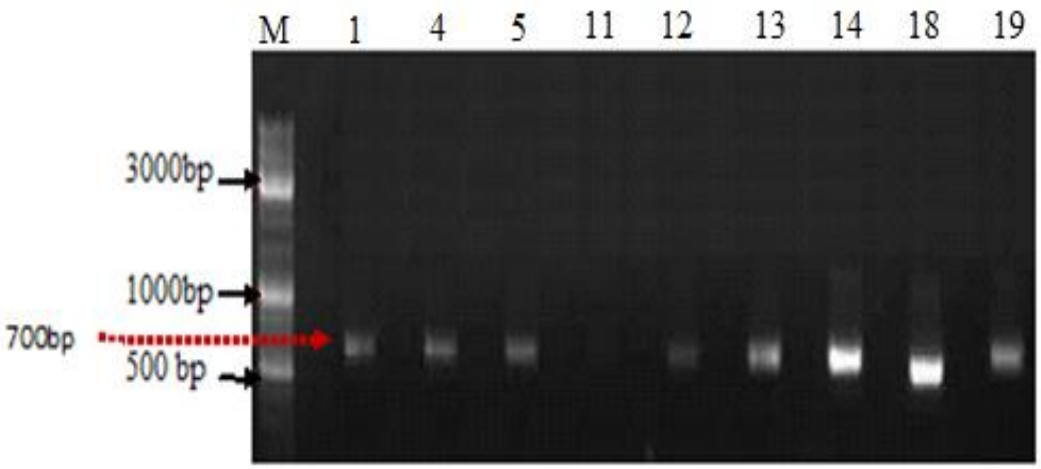


Plate 3.11: PCR amplification of 18S rRNA from fungal isolates on a 1.0% agarose gel

Key: Lane 1, 4, 5, 12, 13, 14, 18, and 19 bands represents fungal isolates namely; *Trichoderma viride, Trichoderma spirale, Neosartorya pseudofischeri, Neosartorya aureola, Aspergillus flavus, Aspergillus terreus, Penicillium griseofulvum* and *Trichoderma longibrachiatum* respectively. Lane 11 was not sequenced since it was negative for amplification. A 3kb DNA ladder was used as a molecular size marker.

3.3.2 Affiliation of 18S rRNA gene sequences of fungal isolates

Eight fungal isolates from oil contaminated soils from informal automobiles were characterized on the basis of phylogenetic analysis. Figure 3.2 shows evolutionary relationship partial 18S rRNA gene sequence of isolates and known fungal species. The accession numbers in bold red are fungal isolates that biodegraded engine oil while the ones in bold black are accession numbers for the fungal isolates that did not biodegrade the oil in the present study (Figure 3.2) Comparison of the 18S rRNA gene sequences to known fungal sequences in the Genbank database using BLAST analysis indicated sequences of more than 97% as shown in the Table 3.2. Isolate 019 [KP296146], 001[KP296139] and 004[KP296140] were closely affiliated with the members from the genus *Trichoderma* with percentage similarity of 98% (Table 3.2). However, these three fungal isolates formed separate sub clusters.

Isolate 018 [KP296145] was closely affiliated with *Penicillium griseofulvum* and had a single sub cluster with a percentage similarity of 99% (Figure 3.2). Isolate 014 [KP296144] and 013 [KP296143] were closely affiliated with members from the genus *Aspergillus* having a percentage similarity of 99% and 100% respectively (Table 3.2). They formed separate sub clusters (Figure 3. 2). Isolate 005 [KP296141] and 012 [KP296142] were closely affiliated with members from the genus *Neosartorya*, with a percentage similarity of 99% (Table 3.2). However, the former isolate formed a single sub cluster (Figure 3.2). There were only two genera that biodegraded engine oil namely; *Penicillium* and *Aspergillus* having percentage similarity of 99% and 100% respectively.

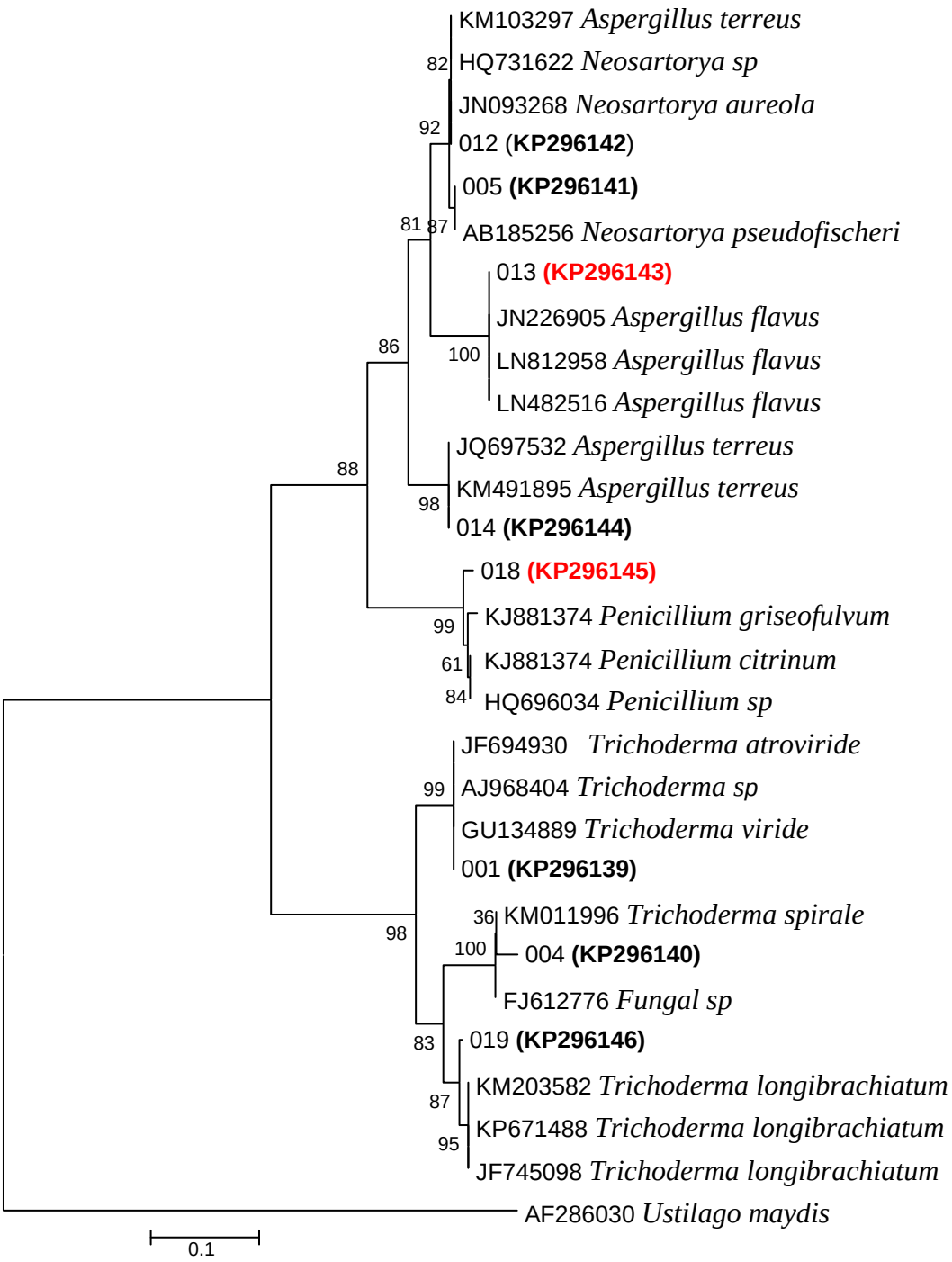


Figure 3.1: Phylogenetic tree.

The nucleotide sequences obtained were aligned with a known fungal isolate *Ustilago maydis* (Accession number) AF286030 (Quadbeck-Seeger *et al.*, 2000) from GeneBank database to show the different fungal classifications. The numbers in bold are accession numbers for the fungal isolates that biodegraded the engine oil. The scale bar indicates approximately 10% sequence difference.

Table 3.2: Taxonomic affiliation of fungal isolates from oil contaminated soils in Thika Town.

ISOLATE CODE	ACCESSION NO.	TAXONOMIC AFFILIATION	ACCESSION NO.	% ID
1	KP296139	*Trichoderma viride*	GU134889	99%
4	KP296140	*Trichoderma spirale*	KM011996	97%
5	KP296141	*Neosartorya pseudofischeri*	AB185256	99%
12	KP296142	*Neosartorya aureola*	JN093268	99%
13	KP296143	*Aspergillus flavus*	JN226905	100%
14	KP296144	*Aspergillus terreus*	KM491895	99%
18	KP296145	*Penicillium griseofulvum*	KJ881374	99%
19	KP296146	*Trichoderma longibrachiatum*	KM203582	98%

3.4 Biodegradation potentials of *Aspergillus flavus* (Code 13) and *Penicillium griseofulvum* (Code 18).

Bacto- Bushnell Hass broth changed colour from blue to colourless meaning that the isolates responsible might be potential hydrocarbon oxidizers. Out of eight isolates, only *Aspergillus flavus* (Code 13) and *Penicillium griseofulvum* (Code 18) had their broth change colour from deep blue to colourless. Plate 3.12 to Plate 3.16 shows flasks for unspent and spent before and after biodegradation. They were further analyzed using GC- MS (HRGC 400B - MSQ12 Konic-Spain). *Aspergillus flavus* (Code 13) and *P. gresiofulvum* (Code 18) biodegraded oil from a higher concentration to below detectable limit. *Aspergillus flavus* (Code 13) could not fully biodegrade decane, undecane and tridecane from a concentration of 5.48 to 0.23, 18.14 to 0.13 and 14.22 to 0.11 mg/l to below detectable limit respectively. *Penicillium gresiofulvum* (Code 18) could also not fully biodegraded 1-Ethylidene-1h –Indene from a concentration of 0.29 to 0.17mg/l to below detectable limit (Table 3.3).

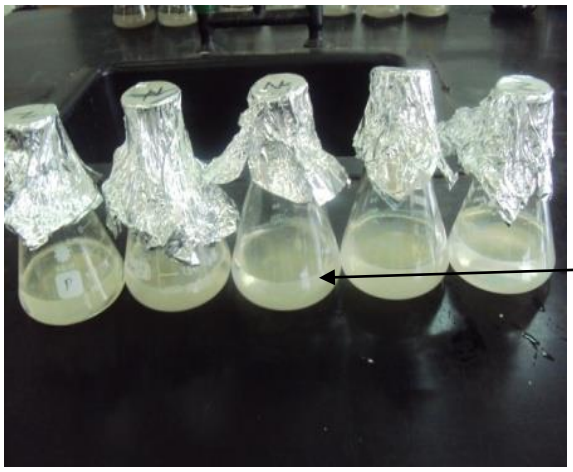
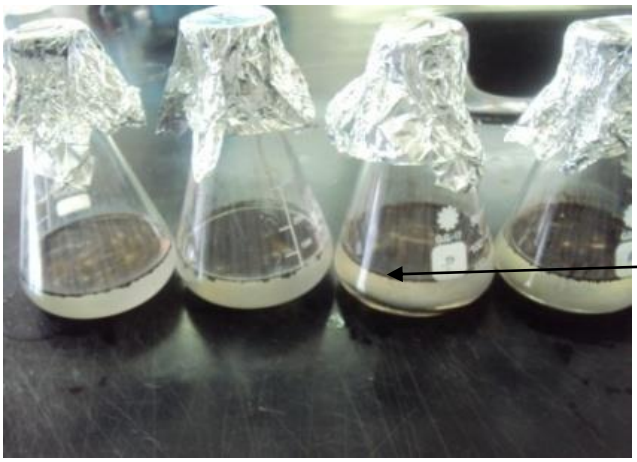
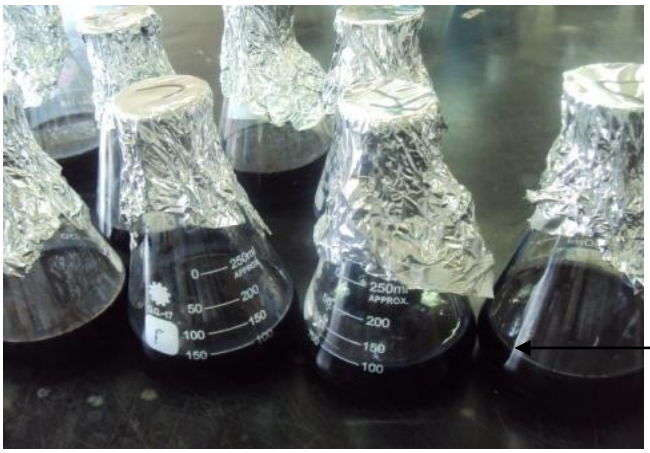


Plate 3.12: Flasks bearing unspent engine oil before biodegradation



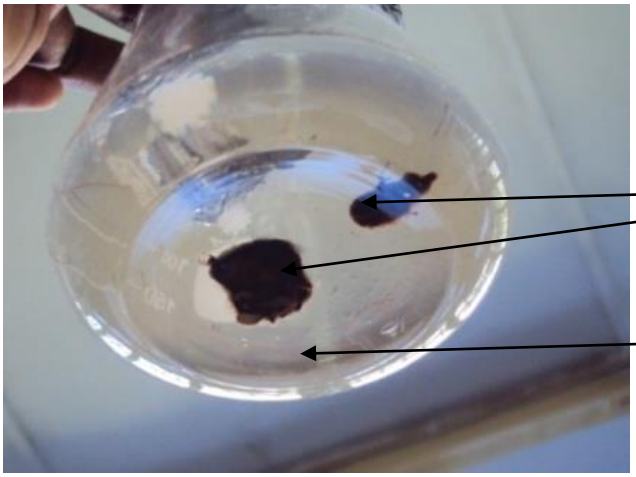
Spent engine oil in the media

Plate 3.13: Flasks bearing spent engine oil before biodegradation



Dark blue colour before biodegradation

Plate 3.14: Flasks bearing redox reagent, oil and fungi before biodegradation



…Plate 3.15: Flask showing biodegraded unused oil by *Penicillium griseofulvum* (Code 18)

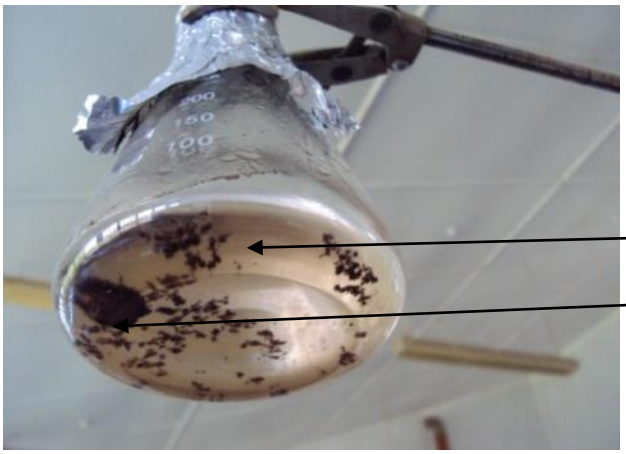


Plate 3.16: Flask showing biodegraded used oil by *Aspergillus flavus* (Code 13).

Table 3.3: *In vitro* biodegradation of compounds of *Penicillium gresiofulvum* (Code18) and *Aspergillus flavus* (Code13).

Compound name	**Concentration in (mg/l) of unused engine oil by *Penicillium griseofulvum***		**Compound name**	**Concentration in (mg/l) of used engine oil by *Aspergillus flavus***	
	Before Biodegradation	**After Biodegradation**		**Before Biodegradation**	**After Biodegradation**
3-Ethyltoluene	2.24	0	Decane	5.48	0.23
			4,6,8-Trimethyl-1-nonene		
Benzocyclopentane	1.69	0		4.57	0
Dihydrodicyclopentadiene	1.98	0	Undecane	18.14	0.13
1,4-Dimethyl-2-Ethylbenzene	0.69	0	Dodecane	18.99	0
1,2-Dimethyl-4-Ethylbenzene	1.31	0	Trans-3-Dodecene	2.92	0
			4-Ethylundecane		
1,2,4,5-Tetramethylbenzene	1.13	0		9.03	0
4-Methylindan	0.98	0	Tridecane	14.22	0.11
			5Z)-5-Tridecene		
2-Methylindan	1.64	0		1.65	0
Tetradecane	2.58	0	Tetradecane	11.37	0
Pentadecane	2.79	0	Pentadecane	3.56	0
1-Ethylidene-1h-Indene	0.29	0.17			
Benzocyclohexane	1.60	0			
Naphthalene	0.81	0			
Phenylbenzene	0.98	0			
1-Ethylnaphthalene	0.09	0			
1,3-Dimethylnaphthalene	0.50	0			
9h-Fluorene	1.08	0			
Hexadecane	1.64	0			

3.5 Physiological characterization of fungal isolates

3.5.1 Fungal mycelial growth at different temperatures

When different organisms were grown at different temperatures, they showed different radial growths. *Trichoderma spirale* had optimal growth at 30^{o}C and poor growth at 40^{o}C. There was no growth at 50^{o}C. *Trichoderma longibrachiatum* had optimal growth at 30^{o}C and no growth at 40^{o}C and 50^{o}C. *Neosartorya pseudofisheri* had optimal growth at 30^{o}C and minimal growth at 40^{o}C. No growth was observed at 50^{o}C. *Penicillium griseofulvum* had optimal growth at 40^{o}C and minimal growth at 30^{o}C. No growth was observed at 50^{o}C. *Trichoderma viride* grew only at 30^{o}C and no growth at 40^{o}C and 50^{o}C. *Aspergillus flavus* grew at all temperatures. Optimal growth was observed 40^{o}C, minimal growth at 30^{o}C and poor growth at 50^{o}C. *Aspergillus terreus* had optimal growth at 40^{o}C, and minimal growth at 30^{o}C. No growth was observed at 50^{o}C. *Neosartorya aureola* had optimal growth at 30^{o}C and a very minimal growth at 40^{o}C. No growth was observed at 50^{o}C. Therefore, there was no significance difference $p > 0.05$, 0.997973 (Supplementary Table 1) at the appendices on radial growth for fungal isolates at three different temperatures (Fig. 3.2).

3.5.2 Fungal mycelial growth at different pH

There was optimal growth at pH 5, 7 and 9 by *Trichoderma spirale, Trichderma longibrachiatum, Neosartorya pseudofischeri* and *Aspergillus terreus.* Minimal growth was noted by *Penicillium griseofulvum, Trichoderma viride* and *Aspergillus flavus* at pH 5, 7 and 9. Poor radial growth was noted by *Neosartorya aureola* at pH 5, 7 and 9. However, the best growth was at pH 9 while poor growth was at pH 5. There was significance difference $p<0.05$ (Supplementary Table 2) at the appendices on radial growth for fungal isolates at three different pH (Fig. 3.3).

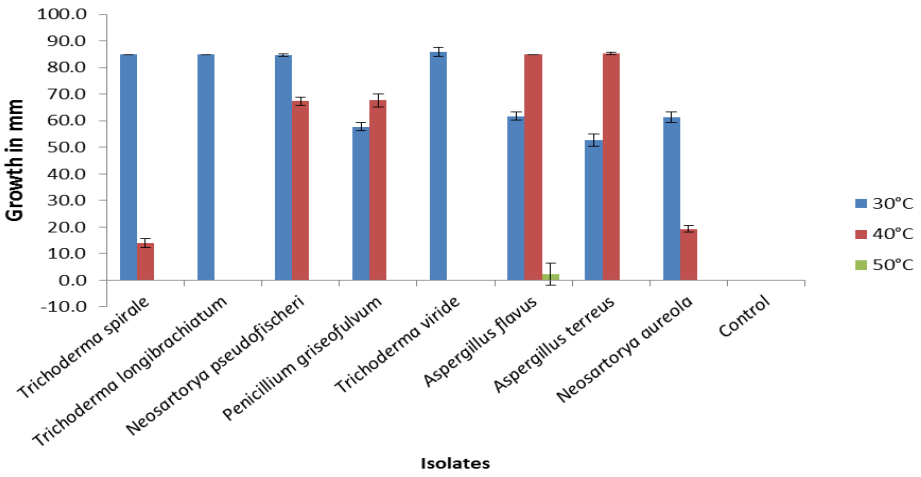


Figure 3.2: Effect of temperature on fungal growth

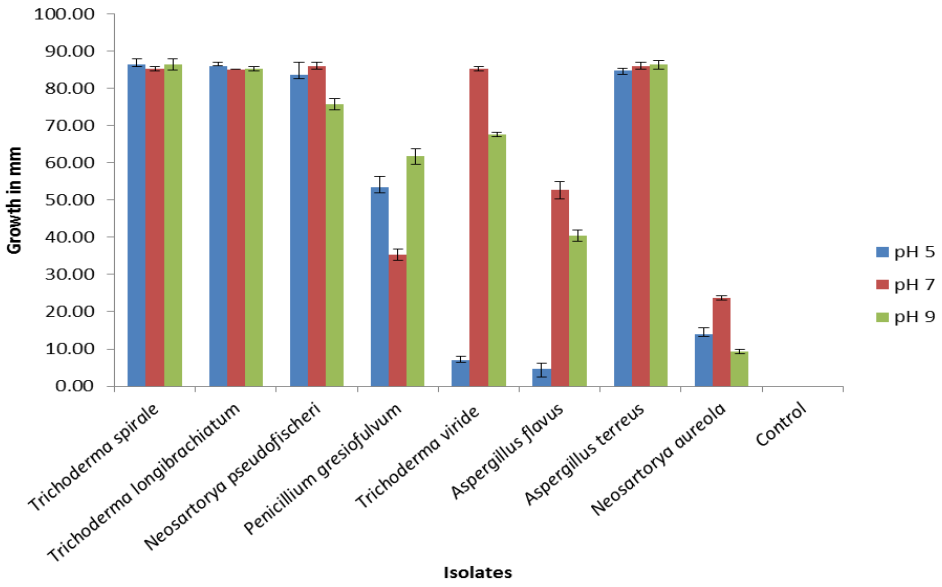


Figure 3.3: Effect of pH on fungal growth

3.6 Enzymatic activity of fungal isolates

All isolates were positive for amylase hence starch was hydrolyzed. Protease production for all isolates was observed and so skim milk was hydrolyzed. Two were negative for lipase/esterase production while the rest six were positive. This shows that the six positive isolates hydrolyzed the olive oil while the rest two could not. All isolates tested positive for cellulose and so cellulose was well hydrolyzed (Table 3.4). Plate 3.15 shows hydrolysis of skim milk by *Aspergillus flavus* while Plate 3.16 shows hydrolysis of starch by *Neosartorya pseudofischeri*.

Table 3.4: Production of Extra- Cellular enzymes by fungal isolates

ISOLATE CODE	ISOLATE IDENTITY	AMYLASES	PROTEASES	LIPASES/ESTERASES	CELLULASES
1	*Trichoderma viride*	+	+	_	+
4	*Trichoderma spirale*	+	+	_	+
05	*Neosartorya pseudofischeri*	+	+	+	+
12	*Neosartorya aureola*	+	+	+	+
13	*Aspergillus flavus*	+	+	+	+
14	*Aspergillus terreus*	+	+	+	+
18	*Penicillium griseofulvum*	+	+	+	+
19	*Trichoderma longibrachiatum*	+	+	+	+

Key: '(+)' Denotes hydrolysis and '(-)' Denotes no hydrolysis.

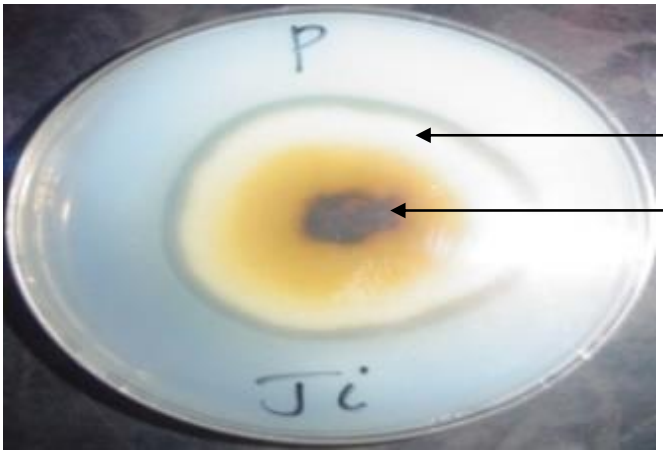


Plate 3.17: Skim milk hydrolysis by *Aspergillus flavus*

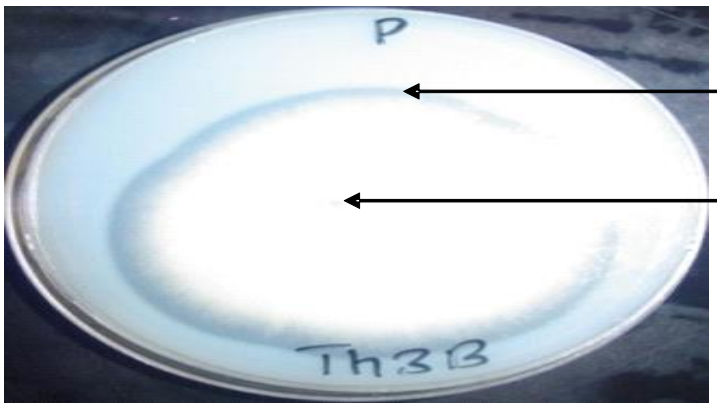


Plate 3.18: Starch hydrolysis by *Neosartorya pseudofischeri*

CHAPTER FOUR

4.0 DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

Eight isolates namely; *Trichoderma viride, Trichoderma spirale, Neosartorya pseudofischeri, Neosartorya aureola, Aspergillus flavus, Aspergillus terreus, Penicillium griseofulvum* and *Trichoderma longibrachiatum* were recovered from oil contaminated soils . The presence of these fungi in soil samples indicated that, the isolates were able to exist in the oil contaminated environment while those that could not survive in this environment being eliminated by the unfavorable conditions caused by the oil (Adekunke & Adebambo, 2007).

Molecular characterization and phylogenetic analysis showed all the isolates belonged to the same domain of Eukaryota, phylum of *Ascomycot*a and subphylum of *Pezizomycotina.* The phylogenetic tree also revealed that the fungal isolates belonged to four different genera namely; *Trichoderma, Neosartorya, Aspergillus and Penicillium.* The above mentioned genera were supported through morphological characterization. However, *Trichoderma* genera differed from the other three genera due to the hierarchical classification. The difference was noted from the class upto the family. This included, (classs) *Sordariomycetes,* (subclass) *Hypocreomycetidae*, (order) *Hypocreales,* and (family) *Hypocreaceae.* Three genera namely *Neosartorya, Aspergillus and Penicillium* had the same hierarchical classification of (class) *Eurotiomycetes,* (subclass) *Eurotiomycetidae,* (order) *Eurotiales* and family of *Aspergillaceae* (Hibbet *et al.,* 2007).

Comparison of the 18S rRNA gene sequences to known fungal sequences in the Genbank database using BLAST analysis indicated sequences of more than 97% as shown in the Table 3.2. Isolate 019 [KP296146], 001[KP296139] and 004[KP296140] were closely affiliated with the members from the genus *Trichoderma* with percentage similarity of 98%. However, these three fungal isolates formed separate sub clusters (Husaini *et al.,* 2008).

Isolate 018 [KP296145] was closely affiliated with *Penicillium griseofulvum* and had a single sub cluster with a percentage similarity of 99% (Zhu *et al.,* 2001). Isolate 014 [KP296144] and 013 [KP296143] were closely affiliated with members from the genus *Aspergillus* having a percentage similarity of 99% and 100% respectively (Iranzo *et al.,* 2001). They formed separate sub clusters. Isolate 005 [KP296141] and 012 [KP296142] were closely affiliated with members from the genus *Neosartorya*, with a percentage similarity of 99% (Obire *et al.,* 2008). However, the former isolate formed a single sub cluster. There were only two genera that biodegraded engine oil namely; *Penicillium* and *Aspergillus* having percentage similarity of 99% and 100% respectively (Smita *et al.,* 2012).

Out of eight isolates recovered from the oil contaminated environment namely; *Trichoderma viride, Trichoderma spirale, Neosartorya pseudofischeri, Neosartorya aureola, Aspergillus flavus, Aspergillus terreus, Penicillium griseofulvum* and *Trichoderma longibrachiatum,* only *Penicillium griseofulvum* and *Aspergillus flavus* showed potential to biodegrade engine oil. The use 0.1% of Tween 80 by the two mentioned isolates facilitated the transport of the oil to fungi cells and enhanced the metabolism of the hydrocarbon (George-Okafor *et al.,* 2005). The ability of the two isolates to produce a colour change in the Bacto Bushnell – Hass broth medium is presumably due to the reduction of the indicator 2, 6-dichlorophenol indophenols (DCPIP) by the oxidized products of hydrocarbon degradation. This is however rapid, easy and a cost effective method that can be used to detect fungal metabolism from hydrogen and carbon (Buchanan, 1991).

The analysis of the biodegradative ability of the isolated fungi showed that the organisms maximally utilized engine oil as most of the compounds were below detectable limit at the end of the experiment. This might be due to the fact that the fungal isolates used hydrocarbons as a substrate for growth by dismantling the long chain of hydrogen and carbon, hence converting oil into simpler forms of products that can be absorbed for the growth and nutrition of the fungi (Adekunke & Adebambo, 2007). Naphthalene an unsaturated aromatic hydrocarbon which is carcinogenic was fully biodegraded. Saturated hydrocarbon like decane is straight

chain and was not fully biodegraded. This could be due to the factors like concentration, composition, environmental factors and biological state of the organism at the time of contamination. These factors could also be probable reason as to why the rest six fungal isolates could not biodegrade engine oil in the present study (Cerniglia & Sutherland, 2001).

The results of the present study shows that *P. griseofulvum* had higher biodegradation potential compared to *A. flavus* since only one compound could not be fully biodegraded by the former and three compounds by the latter isolate. This difference could be as a result of exhaustion of nutrients, difference in growth rates, and size of the molecules or release of toxic material into the medium. Although developed countries have commercially produced fungal cultures for oil cleanup, this is less common in developing countries. One region subject to frequent detrimental oil spills, and lacking access to commercially produced cleanup strains, is the Niger Delta. This region may benefit from development of indigenous fungi for bioremediation. It is therefore recommended that the crude oil which cannot be recovered after a spill should therefore be subjected to bioremediation using fungi and fungal products such as their enzymes (Azaiki, 2009).

A large number of studies from Nigeria have been reported on the biodegradation of hydrocarbons using strains of *Penicillium*, *Rhizopus* and *Aspergillus* (Kapoor *et al*., 1999; Say *et al*., 2004; Ahmad *et al*., 2005). White-rot fungi like *Pleurotus* species have been known for their ability to degrade lignin, a non-repeating structural polymer found in woody plant and this ability enables them to degrade xenobiotic pollutants (Bumpus & Aust, 1987). Earlier studies by Okerentugba & Ezeronye (2003) from Nigeria demonstrated that *Penicillium* spp, *Aspergillus* spp. and *Rhizopus* spp. were capable of biodegrading hydrocarbons. The radial growth for the fungal isolates under different pH in this study was significantly different ranging from neautral to alkaline. The results of the present study is partly inconsistent with those obtained by Atlas and Bragg, 2009.

There was significant difference observed in the radial growth of the fungal isolates under different temperatures. This indicates that, higher temperatures increase the rate of hydrocarbon metabolism to an optimal radial growth at 30^{o}C and 40^{o}C for most isolates. Venosa and Zhu (2003) reported that, temperature plays very important roles in biodegradation of petroleum hydrocarbons, by its direct effect on the chemistry of the pollutants, effect on the physiology and diversity of the microbial communities. Highest degradation rates generally occur in the range of 30^{o}C to 40^{o}C in soil environments, 20^{o}C to 30^{o}C in some freshwater environments, and 15^{o}C to 20^{o}C in marine environments (Bossert & Bartha 1984). The optimum temperature for *A. flavus* to grow is 37^{o}C, but fungal growth can be observed at temperatures ranging from 12 to 48^{o}C. Such a high optimum temperature contributes to its pathogenicity in humans. The ability of *A. flavus* to survive in harsh conditions allows it to easily out-compete other organisms for substrates in the soil or in the plant (Gibson *et al.,* 1994). This is the reason why *A. spergillus flavus* could grow at 50^{o}C from the present study.

The radial growth for the fungal isolates under different pH in this study was significantly different ranging at neutral pH. The results of the present study are partly consistent with those obtained by Atlas and Bragg, 2009. Microorganisms have evolved several enzymes for biodegradation including cellulase, xylanase and ligninolytic enzymes (Bogan & Lamar, 1996). The ability of the fungal isolates to produce amylase, cellulose, protease and lipase enzymes, demonstrate their ability to biodegrade enzymes containing substrate. This supports the view that, fungi play a role in the biodegradation of oil contaminating soils in the environment. Studies by Bogan and Lamar (1996) indicate that biodegradation by fungi could be due to their massive growth and enzyme production responses during their growth phases. The main industrial use of fungi has been associated with the production of fermentation products, including antibiotics, enzymes and a range of biochemical. Ethanol, citric acid, gluconic acid, itaconic acid, amino acids, vitamins, nucleotides and polysaccharides provide examples of primary metabolites produced by fungi. Antibiotics such as penicillin, the cephalosporins, fusidic acid and griseofulvin are important secondary metabolites (Namikoshi *et al.,* 2002). Fungi are well known as

a source of antibiotics but new therapeutic compounds with novel pharmacological activities have also been developed in recent years (Jensen & Fenical, 2000). One such example is the cyclosporins first isolated from *Tolypocladium inflatum* in 1976 as antifungal. Cyclosporin A is currently the most widely used drug for preventing rejection of human organ transplants (Borel, 2002).

After mass production of the fungal isolate recovered, the fungus can be ground and formulated with adequate carriers or extenders either in the powdered or liquid form using Tween 80 which enhances the degradation process. In order to prevent run-offs, 'inert' stickers or adhesives (such as molasses, and corn syrup) may be incorporated into the formulation (Atlas & Bragg 2009). A good sticker combined with charcoal can serve as a protectant. This will reduce the effects of ultra-violet light, desiccation and other detrimental environmental factors. While carrying out the study, the oil contaminated plots to be treated should first be tilled to loosen the soil (Okerentugba & Ezeronye 2003). Thereafter, the loosened soil should be enriched with adequate nutrients necessary for the growth of the organism before applying the formulated inoculants which must be properly mixed with the soil. Periodic moistening of the soil may be necessary to avoid excessive drying of the contaminated soil (Uzoamaka *et al.*, 2009).

4.2 Conclusions

A total of eight fungal isolates were isolated and characterized morphologically from polluted soils namely, *Trichoderma viride* (Code 1), *Trichoderma spirale* (Code 4) *Neosartorya pseudofischeri* (Code 5), *Neosartorya aureola* (Code12), *Aspergillus flavus* (Code 13), *Aspergillus terreus* (Code 14), *Penicillium griseofulvum* (Code 18) and *Trichoderma longibrachiatum* (Code 19). The eight isolates were identified through the comparison of the 18S rRNA gene sequences to known fungal sequences in the Genbank database using BLAST analysis indicating similarity of more than 97%. However, the percentage similarity for the isolates that biodegraded oil namely, *Penicillium griseofulvum* (Code 18) and *Aspergillus flavus* (Code 13) were 99% and 100%. This means that the isolates that biodegraded engine oil were closely related with those from the NCBI.

Screening of the eight fungal isolates for biodegradation was done and only *Aspergillus flavus* and *Penicillium griseofulvum* was found to biodegrade seven and seventeen oil compounds respectively. The optimum growth temperature range for the eight fungi was 40^{o}C. The optimum pH of the eight isolates was 7. The eight fungi were also screened for extracellular enzymes namely; amylase, protease, lipase/esterase and cellulose enzymes, demonstrating their ability to biodegrade oil containing substrate. This study will contribute to the database on locally available fungal diversity and their ecology and also increase knowledge of the fungi involved in biodegradation of oil in Thika town.

4.3 Recommendations

From the findings in this study, it can be recommend that;

1. Carry out *ex-situ* experiment using *Penicillium griseofulvum* and *Aspergillus flavus* to biodegrade spent and unspent engine oil.
2. Further research using different fungal isolates like the ones isolated from Niger delta and test whether the compounds that failed to fully biodegrade will do so.

3. Carry out test on other different environmental parameters such as oxygen, nutrients, salinity, and pressure and water to see if they affect biodegradation of oil by fungi.

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APPENDICES

Appendix A: Supplementary Table 1: ANOVA for the eight fungal isolates at different temperatures.

ANOVA

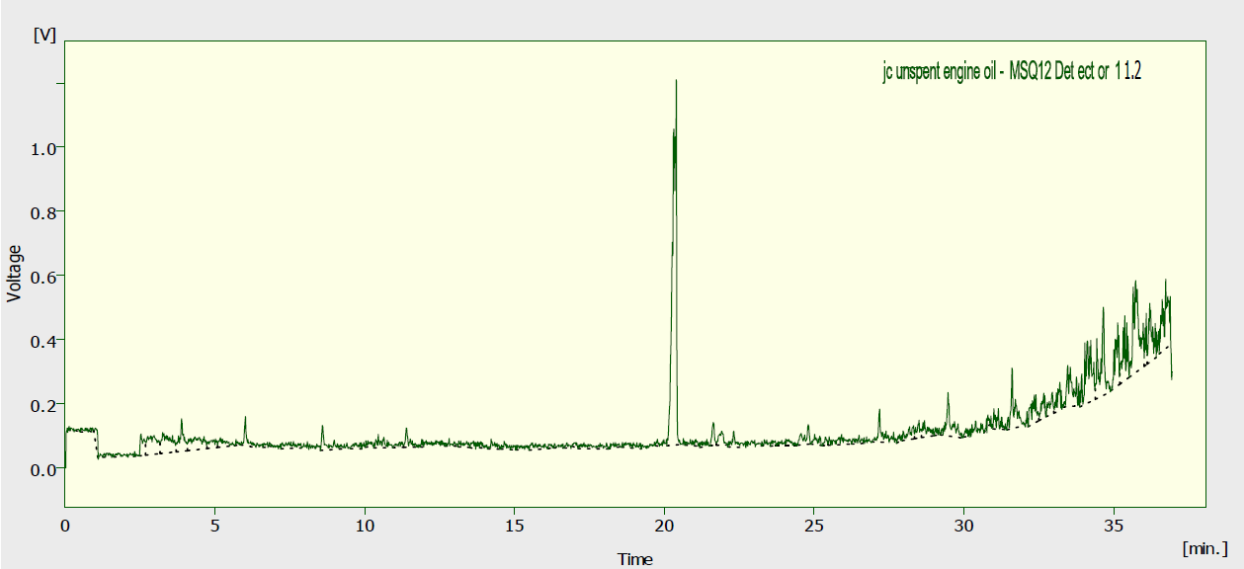
Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Temp	6.740741	2	3.370371	0.002029	0.997973	3.168246
Isolate	17961.56	8	2245.194	1.351662	0.238862	2.115223
Interaction	22.37037	16	1.398148	0.000842	1	1.834629
Within	89697.33	54	1661.062			
Total	107688	80				

Appendix B: Supplementary Table 2: ANOVA for the eight fungal isolates at different pH.

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
pH	2631.135802	2	1315.568	670.1952	7.52E-39	3.168246
Isolate	77811.35802	8	9726.419	4954.969	1.64E-74	2.115223
Interaction	12817.75309	16	801.1096	408.1124	2.41E-50	1.834629
Within	106	54	1.962963			
Total	93366.24691	80				

Appendix C: Chromatogram of unused engine oil biodegraded by *Penicillium griseofulvum*.



Appendix D: Chromatogram of used engine oil biodegraded by *Aspergillus flavus*.