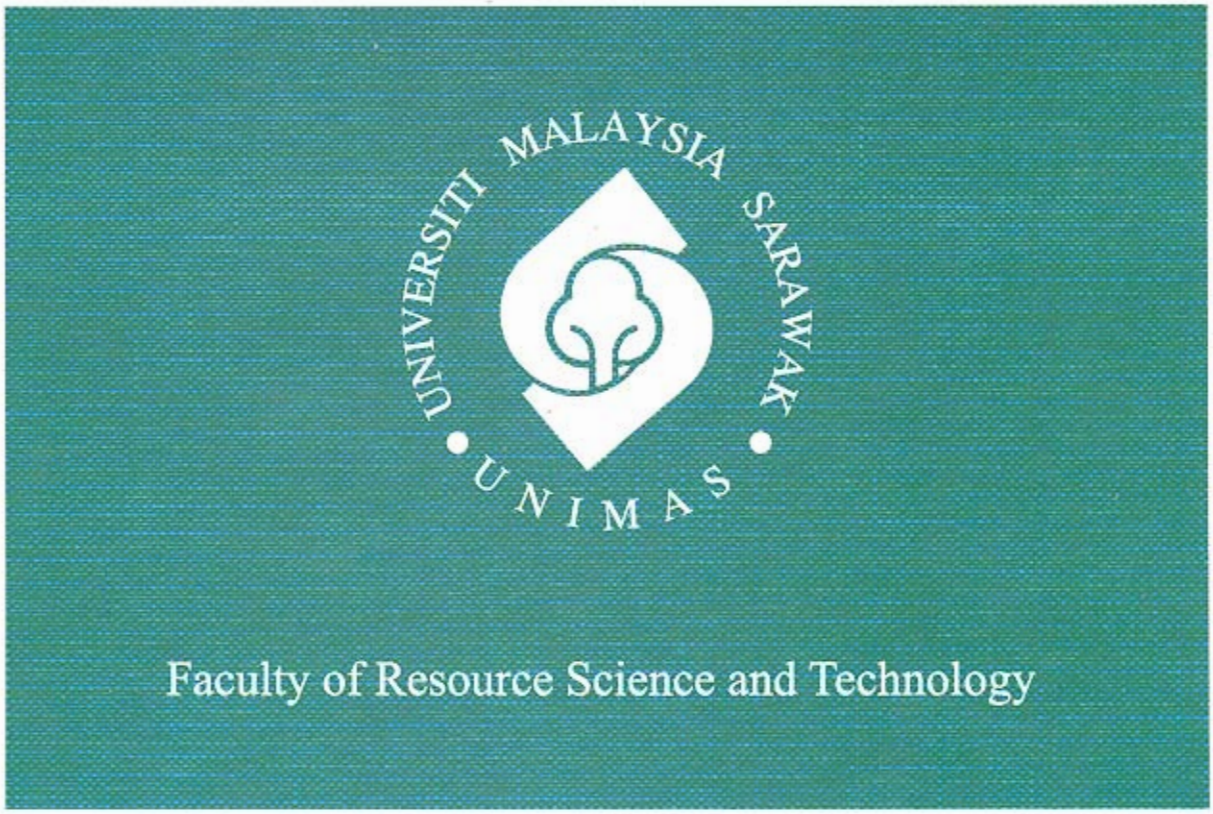

BIODEGRADATION OF USED MOTOR OIL BY *ASPERGILLUS SPP.* ISOLATED FROM OIL CONTAMINATED SOIL

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Bachelor of Science with Honours
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List of Abbreviations

A.flavus	*Aspergillus flavus*
A.versicolor	*Aspergillus versicolor*
Cm	Centimeter
g	gram
LiP	Lignin Peroxidase
L	Liter
mL	mili Liter
mM	miliMolar
MnP	Manganese Peroxidase
OD	Optical Density
PAH	Polycyclic Aromatic Hydrocarbon
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
sp	Species
µmol	micromol

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ABSTRACT

Aspergillus sp is a common hydrocarbon degrading fungi isolated from oil contaminated site. The present study looks on biodegradation ability of *Aspergillus flavus* and *Aspergillus versicolor* on used motor oil and their morphological changes during the degradation process in batch culture with shaking and without shaking. Hydrocarbon analyses were performed by measuring lignolytic enzyme activity and gravimetric analysis, whereas morphological analyses were assessed through biomass determination and microscopic observation. Possible relationship was drawn between the degradation ability and biomass production. Lignin peroxidase were secreted the most among the lignolytic enzyme and lignin peroxidase and manganese peroxidase were significant whereas laccase is not significant for *Aspergillus* sp. *Aspergillus versicolor* had higher degradation percentage with 97.01% degradation of aliphatic hydrocarbon,F1 and 97.63% degradation of polycyclic aromatic hydrocarbon,F2 in the batch culture with shaking. The biomass production was significant for *Aspergillus* sp and shows a similar morphological condition for each week in all the culture throughout the degradation process. The biomass production and degradation ability of *Aspergillus* sp is not significant therefore not linearly related. Further research can be conducted by assessing non-lignolytic enzyme activity and use more accurate growth measurement to correlate the morphological changes and the degradation ability.

Key Words: Biodegradation, Used motor oil, *Aspergillus flavus*, *Aspergillus versicolor*

ABSTRAK

Aspergillus spp. *ialah fungi pegurai hidrokarbon yang biasa didapati di kawasan yang tercemar dengan minyak. Dalam kajian in, keupayaan Aspergillus flavus dan Aspergillus versicolor mengurai minyak motor terguna dan pemerhatian akan perubahan morfologi semasa proses penguraian di dalam kultur dengan goncangan dan tanpa goncangan dijalankan. Analisis hidrokarbon dijalankan melalui pengukuran aktiviti enzim lignolitik dan analisis gravimetrik manakala analisis morfologi dibuat melalui mengambil berat mycelium dan pemerhatian mikroskopik. Sementara itu satu hubungan antara berat mysilia dan kebolehan mengurai oleh Aspergillus* spp. *telah dilakukan. Lignin peroksida adakah enzim yang paling tinggi dirembes dan analisis Anova menunjukkan lignin peroksida dan manganese peroksida adalah signifikan manakala laccase tidak signifikan bagi Aspergillus* spp. *Aspergillus versicolor mempunyai peratus penguraian yang tinggi iaitu 97.01% penguraian bagi hidrokarbon aliphatik, F1 dan 97.63% penguraian bagi hidrokarbon aromatik, F2 dalam kultur dengan goncangan. Berat mysilia didapati signifikan bagi Aspergillus* spp. *dan ia menunjukkan perubahan morfologi yang sama semasa proses penguraian bagi setiap minggu dalam kedua-dua kultur. Satu hubungan yang selari tidak dapat disimpulkan antara berat mysilia dan kebolehan mengurai oleh Aspergillus* spp. *dimana kaitan antara kedua-dua faktor didapati tidak signifikan. Kajian lanjut boleh dijalankan untuk menilai aktiviti enzim bukan-lignolitik yang dirembeskan samasa proses penguraian dan kaedah mengukur pertumbuhan yang lebih jitu boleh digunakan supaya dapat memperoleh satu hubungan antara perubahan morfologi dengan kebolehan mengurai.*

Kata Kunci: Biodegradasi, minyak motor terpakai, Aspergillus flavus, Aspergillus versicolor

CHAPTER 1.0

Introduction

Petroleum is a viscous liquid that are mainly composed of hydrogen and carbon. In the presence of large concentration of petroleum, it is considered toxic to many organisms including human. The petroleum and petroleum products have contributed large extent of hydrocarbon contamination to both the soil and marine environment. In simplification, oil pollution from industrial sources and other activities possess hazardous to terrestrial and marine ecosystem (Emtiazi *et al*., 2005). Soil, lakes, rivers, and seas are known to highly be contaminated with different toxic compounds such as aliphatic hydrocarbon, polycyclic aromatic hydrocarbon (PAH) and asphaltene.

The sequences of events of oil spillage that takes over the years such as supertanker Torrey Canyon sank in English Channel (Atlas, 1981) and spillage of more than 200000 barrels of crude oil taken from Exxon Valdez in Prince William Sound, Alaska has possessed a great threat to the environment. Subsequently it has leads the science towards development of biodegradation process as a resolution to this problem. Biodegradation in natural ecosystem is a complex process that involves enzymatic degradation of the complex mixture into smaller compounds by the living organisms such as fungi and bacteria.

Atlas (1981) has stated that in 1946, Claude E Zobell has discovered many microorganisms that able to utilize hydrocarbon as sole source of energy and carbon. Fungi, bacteria and algae are examples of the microorganism that are able to survive in the oil-contaminated environment and secrete specific enzymes that are capable to degrade available

hydrocarbon which serves as energy source for them. According to Lindstorm *et al*. (1991) and Madsen (1991), upon realizing the role of microorganism in biodegradation of hydrocarbon, much attention has been given to the study especially emphasizing the degradation potential of microorganism for bioremediation process in future. This is vital as the environment is rapidly being polluted especially with hydrocarbon-containing compounds. Therefore disposing these pollutants is necessary. The introduction of bioremediation process as an application of biodegradation process is effectively applied in cleaning the oil spillage either in soil or sea (Singh, 2006).

In this study, isolate of *Aspergillus flavus* and *Aspergillus versicolor* was studied since *Aspergillus spp.* are the fungi that are most commonly isolated from the soil contaminated with hydrocarbon (Elshafie *et al*, 2008). The *Aspergillus spp.* is analysed for its efficiency of secreting lignolytic enzyme in liquid state during the biodegradation process. Besides that the ability of the *Aspergillus spp.* to degrade hydrocarbon content in the used motor oil were also assessed by conducting simple hydrocarbon analysis that is gravimetric method. Finally the morphological changes in *Aspergillus spp.* during the biodegradation process were also observed. The entire experiment is concentrated on aerobic biodegradation process only.

1.1 Problem Statement

Ability of *Aspergillus spp.* to degrade hydrocarbon in used motor oil and the type of enzyme that assists it in its degradation ability will be one of the problems that will be analysed in this study. In addition to that, the morphological changes in *Aspergillus spp.* during the degradation process are also a problem in this study. Finally the existence of possible relationship between the morphological changes and degradation ability of the *Aspergillus spp.* is also a problem to be analysed in this study.

1.2 Research Objective

Aspergillus spp. is known as hydrocarbon degrading fungi which are commonly isolated from the oil contaminated soil and it able to degrade hydrocarbon components in the oil in order to survive. Therefore the main objective of this study includes:-

(a) To assess the lignolytic enzyme productivity level released by the *Aspergillus* spp. during the degradation process

(b) To analyze the ability of the *Aspergillus spp.* to degrade the hydrocarbon composition in used motor oil

(c) To examine the morphological changes occurs in *Aspergillus spp.* during degradation process in order to draw a possible relationship between morphology changes and degradation process.

CHAPTER 2.0

Literature Review

2.1 Hazards of Petroleum Hydrocarbon

As the world is moving towards higher technology, the level of pollution is also increases and oil contamination is one of the major factors contributing towards the pollution known today. Moreover, the spillages from used motor oil such as diesel and jet fuels have also cause increases on hydrocarbon contamination to the environment. This is due to the increase in utilization of motor oil in accordance with the increase in the number of variety of automobiles and machinery vehicles (Husaini, 2008). According to Hamman (2004) United States is claimed to dispose only 10 percent from eighty billion pounds of hazardous organopollutant safely every year.

Oil contamination can takes place either in soil or sea and their effects to the nature also different. The sea oil contamination poses a great threat to the environment since it directly expose the marine organism to the threat and cause death. The sea oil contaminations are normally resulting from the spillage and accidents occurs during transportation of the oil across sea. A recent oil spillage has took place in June,2001 at Johor, Southern Malaysia where the Indonesian-Registered oil tanker MT Endah Lestari capsized and spilled over 600 metric tons of phenol and large amount of diesel. The spillage has resulted in death of thousands of marine life in the nearby fish farming ground. In contrast the soil contamination by oil cause impact in longer period of time by poisoning food crops, water supplies and so

on. The oil contamination causes nitrogen deficiency and oxygen depletion especially in the deeper layer resulting in the low survival rate for most organisms in this habitat.

Many successful researches have been conducted in isolating and characterizing microorganism such as bacteria and fungi that has potential of degrading hydrocarbon component. For example, recently Elshafie *et al.* (2008) have isolated and characterized fungi from crude oil tar balls on the beaches in Oman that were able to degrade n-alkanes and crude oil. The fields of study on oil pollution are becoming more abundance over the time as the petroleum industry develops and subsequently the number of unavoidable spillages during routine operation as well as accidents during transportation also increases.

2.2 Motor Oil

Motor oil is widely used in present days as the number of different types of automobile and machinery vehicles increases (Husaini *et al.,* 2008). It is a derivative of petroleum hydrocarbon, an organic compound that consists of hydrogen and carbon. Petroleum hydrocarbon is as a complex mixture which can be fractionated into several important classes such as aliphatic fraction, aromatic fraction, polar fraction and asphaltic (Atlas, 1981). Dominguez-Rozado (2003) has stated that according to Hewstone (1994) and Vazquez-duhalt (1989) used motor oil may contains higher percentage of PAH with wider range of aromatic and aliphatic component that has carbon chain length ranging from C_{15} to C_{50} and additives components compared to fresh oil.

2.3 Biodegradation

Biodegradation has a wide range of definition where it is a process involving enzymatic break down of organic substance into useful products by living organism especially microorganism. In general biodegradation can be refered to as a technology that are able to remediate contaminants by toxic or hazardous chemicals including soil contaminated with petroleum hydrocarbon (Choksi, 2003). Two type of biodegradation have been described, which are aerobic biodegradation and anaerobic biodegradation. Aerobic biodegradation requires oxygen during the degradation process whereas anaerobic biodegradation process occurs in the absence of oxygen. Mohd. Tuah (2006) has stated that aerobic degradation is much more preferred over anaerobic degradation since carbon dioxide and water, environmentally acceptable and less expensive products are produce through complete aerobic biodegradation. Figure 1 illustrates the general pathway for fungal biodegradation where the fungi secretes lignolytic enzymes that able to degrade polycyclic aromatic hydrocarbon (PAH) into quinones and finally emits carbon dioxide and water as the final product of a complete degradation.

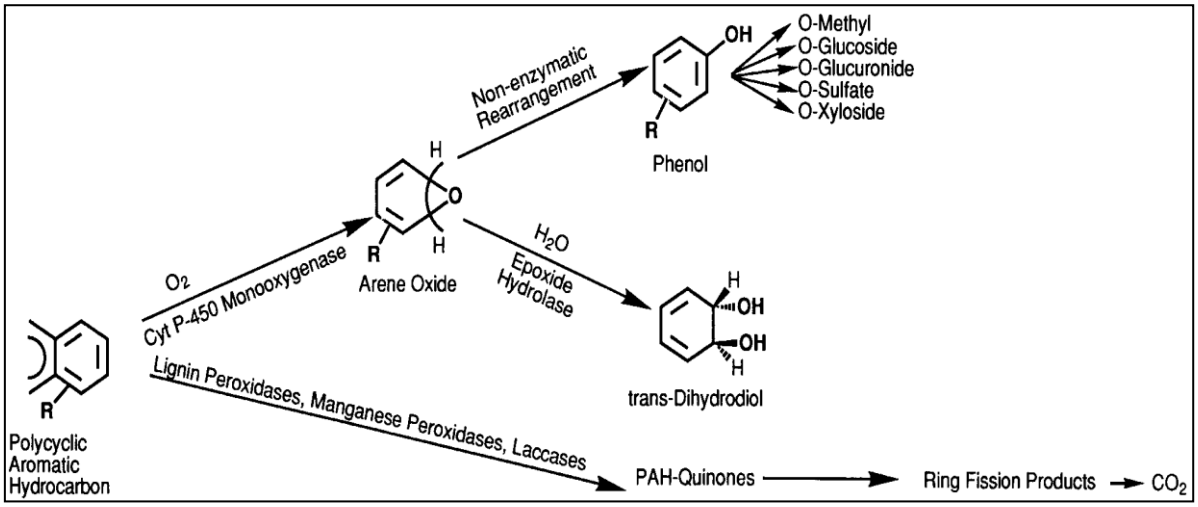

Figure 1: Fungal general biodegradation pathway of polycyclic aromatic hydrocarbon adapted from Cerneglia, C.E. (1997). Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation. *Journal of Industrial Microbiology and Biotechnology*. *19*, 324-333.

Microbial biodegradation is a crucial finding to the environment protection where it able to clean up the oil spillage whether in soil or water. It is more widely practiced since it has less ecological impact towards environment compared to other physical and chemical treatments (Head, 1998; Edington, 1994; cited in Mohd. Tuah (2006). Microbial biodegradation involves a complex series of biochemical reaction (Mohd. Tuah, 2006) which involves depolymerization, demethoxylation, decarboxylation, hydroxylation and aromatic ring opening. The biodegradation potential of bacteria have been long ago recognized. However, fungi have been the subject of recent research (Santos *et al.*, 2004). The fungi role in the bioremediation of oil in soil and sea are enhanced upon realising their capability in detoxifying the oil contaminated area (Singh, 2006). The fungi has many advantage over the bacteria in the degradation process and upon realising it many intense research have been conducted in order to maximize the utilization of fungi in the environmental cleaning process in large scale.

2.4 *Aspergillus spp.*

Aspergillus spp. is a saprophytic fungus that is widely distributed in the marine, fresh water and soil habitat that are contaminated with organopollutant such as oil and as well as in soil, on plants, in the air and on decaying matter. *Aspergillus spp*. can be considered as hydrocarbon degrading fungi since it able to survive in organo pollutant contaminated areas and utilise the available hydrocarbon as its carbon source. Studies have shown that fungi such as *Aspergillus spp.* able to degrade organic compound more efficiently than the traditional bioremediation methods which includes the utilization of bacteria as well (Batelle, 2000; cited in Adekunle *et al.*, 2007). *Aspergillus spp*. as a hydrocarbon degrading fungi has many

advantages over the bacteria in the biodegradation process. One important advantage is that the process is not restricted by hydrophobic environment as in bacteria. Besides, the hyphal movements through the soil allows it to degrade the hydrocarbon compositions that are not reachable by bacteria (April, 1998). *Aspergillus spp.* able to degrade a broad spectrum of hydrocarbon available in the contaminated place since their degradative enzyme machinery consists of a mixture of enzymes such as lignin peroxidase, manganese peroxidase and laccase and the enzymes secreted have a low affinity for their substrates (Annibale *et al*.**,** 2006). Two species of *Aspergillus* are commonly used in the study of used motor oil degradation that is *A.flavus* and *A.versicolor*. *A.flavus and A.versicolor* has been proven as a hydrocarbon degrader by Adekunle & Adebambo (2007) and Sanchez *et al.* (2006) respectively.

2.5 Lignolytic Enzymes

Aspergillus spp. has potential to secrete lignolytic enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase which have lower affinity for substrate specificity (Silva *et al*, 2008). The secreted enzyme enable them to degrade organopollutant especially those with similar structure as to lignin such as polycyclic aromatic hydrocarbon (PAH), aliphatic hydrocarbon and so on (Yamanaka *et al*, 2008). According to pointing (2001) the hydrocarbon degrading fungi uses a non specific free radical mechanism to degrade the hydrocarbon contaminants. The enzyme released degrade the PAH when an electron is added or removed from the ground state and thus get activated to receive or remove electron from other chemicals.

All the lignolytic enzymes are of the oxidoreductase classes of enzyme. The lignin peroxidase is also known as 1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol:hydrogen peroxide oxidoreductase. According to Reddy and Mathew (2001) as cited in Hamman (2004) LiP have higher redox potential than most peroxidases and involve in oxidation of lignin related aromatic compound as well as some non-phenolic aromatic compound. LiP is a hydrogen peroxide dependent enzyme and able to perform its degradation process in the presence of hydrogen peroxide and veratryl alcohol (Goodeve-docker, n.d.).

Manganese peroxidase with the systematic name Mn(II):hydrogen-peroxide oxidoreductase and able to oxidize Mn^{2+} to Mn^{3+} in the presence of hydrogen peroxide. This Mn^{3+} produced able to degrade phenol compound or oxidize a second mediator for the breakdown of non-phenol compounds (Wong, 2008). In addition, it has also been recorded to have the ability to chelate oxalate, which is a small compound that are capable of degrading organopollutant that are out of reach of other fungal enzyme or the hyphae of the fungi itself (Goodeve-docker, n.d.).

Finally the laccase the third lignolytic enzyme is also known with systematic name as benzenediol:Oxygen oxidoreductase. Laccase is a multi-copper bearing lignolytic enzyme function in one-electron oxidation of many phenolic compounds along with the reduction of oxygen to water (Wong, 2008). The general degradation mechanism of this enzyme in the presence of oxygen which is accepted until now is the degradation of phenolic compounds to quinone radical followed by quinines which are finally exposed to further degradation by other enzymes (Goodeve-docker, n.d.).

2.6 Fungal Morphology

The research on fungal morphology is getting importance as it is known to involve in the production of enzymes. Recent study has suggested that the growth process at hyphal tip is correlated with the protein secretion by filamentous fungi (Jiménez-Tobon *et* al., 1997). Besides that it also proven that the pellet size is also influences the extracellular enzyme secretion along with other factors such as different agitation condition and inoculum concentration (Znidarsik, & Pavko, 2001). Generally there are two form of morphology of fungi in a liquid culture that is individual filamentous mycelia which is in a dispersed form or free filaments and spherical colonies called pellets (JimÃnez-Tobon *et al*., 2003). The pellet can be categorized into different groups such as stable spherical or oval agglomerates which contains branched and interwined network of hyphae. The pellet are produce as the spore inoculum into agglomerates of hyphae which are entrapped during germination process (Jimne z-Tobon *et* al., 2003). The pelleted morphology has some advantages over the filamentous growth form such as the problem found in the dispersed mycelia growth as it increases the wall growth and reduces the efficient of mixing and oxygen supply to cells due to increased viscosity of the medium can be overcome in the pelleted form morphology (JimÃnez-Tobon *et al*., 2003). Besides that it also enables the process to be carried on continuously as there is possibility to reuse the biomass (Znidarsik, & Pavko, 2001).

CHAPTER 3.0

Materials and Methods

3.1 Sample Collection

Substrate of this experiment was used motor oil taken from the workshop nearby. The substrate was sterile filtered in prior to use. The *Aspergillus spp.* examined in this study was obtained from UNIMAS Fungal Collection. The *Aspergillus spp.* used is *A.flavus* and *A.versicolor.*

3.2 Fungal Culture Preservation

3.2.1 Short-Term Preservation

A.flavus and *A.versicolor* were transferred into PDA slant separately and preserved in 4^0C in refrigerator for future experimental use.

3.2.2 Long-Term Preservation

Pure cultures of *A. flavus* and *A. versicolor we*re freeze dried by using Nitrogen and preserved at -80^{o}C for future experimental use.

3.3 Working Culture

Working pure culture of *A.flavus* and *A.versicolor* were transferred periodically (every 2 weeks) onto PDA agar slant and preserved at 4^0C in refrigerator for ongoing experiments.

3.4 Preparation of media

PDA is prepared in order to grow the *A. flavus* and *A. versicolor* in order to obtain spores for the inoculation purpose. The spores obtained were then inoculated in mineral salt medium amended with 1% used motor oil to analyze the degradation efficiency of *A. flavus* and *A.versicolor*.

3.4.1 Potato Dextrose Agar

Potato Dextrose Agar (PDA) was used to grow and harvest the spore of *A.flavus* and *A.versicolor*. PDA is prepared by dissolving 39 gram of PDA in 1 liter distilled water. PDA contained 100 mg oxytetracycline/L and 2 mg benomyl/L to control the growth of the bacteria and minimize the development of various hypomycete (moulds). The prepared media was then autoclaved for 15 minutes at 121^0C.

3.4.2 Mineral Salt Media

The mineral salt broth was prepared according to the formula established by Mancera-Lopez *et al.* (2007). The media contains 0.5 g KH_2PO_4, and 0.5 g $(NH4)_2SO_4$, 0.2g KCl, 0.2g

$MgSO_4.7H_2O$ and 0.1 $CaCl_2.2H_2O$ per liter of distilled water. The mineral solution was adjusted to a pH of 6 and sterilized at 121^0C for 15 minutes.

3.5 Trial Biodegradation of *Aspergillus flavus* and *Aspergillus versicolor* on the Used Motor Oil

Trial biodegradation were carried on in order to determine the degrading ability of *A.flavus* and *A.versicolor* in degrading the used motor oil. The degrading ability of each *Aspergillus spp.* were assessed by performing hydrocarbon degradation analysis. 5 pieces of 1cm X 1cm mycelium plugs from the 10^{th} day culture plates of *A. flavus* and *A.versicolor* which are grown on PDA were removed with thin spatula and inoculated in 200 mL mineral salt media amended with 1% (v/v) used motor oil in a 500 mL Erlenmeyer flask. The used motor oil was sterile filtered before used in the experiment. The entire degradation processes were carried out for five weeks with the interval, 1 week. A Negative control is set up as above flask preparation but without the addition of 1% (v/v) used motor oil. The flasks were shaken at 115 rpm at room temperature and the entire experiment is performed in triplicates.

The samples were taken on each week that is week one, two, three, four and five. Approximately equal volume of the sample taken is then transferred into five pre-measured falcon tube and centrifuged at 3000 rpm for 25 minutes. The sample in each tube was separated into 3 layers that are oil layer, supernatant and the pellet. The oil and supernatant are isolated and performed with hydrocarbon analysis and enzyme assay respectively. The dry weight of mycelium harvested was determined and their structural condition was observed in light microscope.