Faculty of Resource Science and Technology

Bioremediation of Hydrocarbon Contaminated Soil using Indigenous Hydrocarbon Degrading Fungi

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May God bless all of you, Thanks!

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List of Abbreviations

cm	Centimere
DNTs	Dinitrotoluenes
Lac	Laccase
LiP	Lignin Peroxidase
ME	Malt Extract
ml	Millilitre
Mn(II)	Manganese (II)
Mn(III)	Manganese (III)
MnP	Manganese Peroxidase
nm	Nanometre
PAHs	Polycyclic Aromatic Hydrocarbons
PDA	Potato Dextrose Agar
PCP	Pentachlorophenols
TNT	Trinitrotoluene
µl	Microlitre
%	Percent
(v/w)	Volume per weight
(w/w)	Weight per weight
°C	Degree Celsius

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ABSTRACT

Hazardous pollutants such as Polycylic Aromatic Hydrocarbons (PAHs) which can be found in motor oil, have strained public concerns as PAHs are toxic, mutagenic and also carcinogenic. Bioremediation has been the best option for converting these hazardous pollutants into less hazardous compounds by utilizing microorganisms. In this research, *Bionectria sp.* was chosen for bioremediation of used motor oil in soil. Time of incubation, percentage of inoculums, moisture content of the soil, and percentage of oil use has been optimized for the bioremediation process. *Bionectria sp.* was inoculated for degrading used motor oil at different percentage, 3 %, 5 %, and 10 % together with different moisture content of soil, 40 % and 60 % for six weeks. Remaining used motor oil extracted out from soil using toluene and the optical density was checked. The best result was given by sample with 8 % (v/w) of inoculum – 60 % of moisture content – 10 % (v/w) used motor oil used with 91 % of the used motor oil degraded.

Keywords: Bioremediation, *Bionectria sp.*, used motor oil, inoculum, and moisture content

ABSTRAK

Bahan pencemar membahaya seperti hidrokarbon aromatik polisiklik (PAHs) yang boleh dijumpai dalam minyak motor, telah membangkitkan kesedaran awam memandangkan PAHs adalah toksik, mutagenik, dan juga karsinogenik. Bioremediasi merupakan pilihan terbaik bagi menukarkan bahan pencemar ini kepada bahan yang kurang mencemarkan alam dengan menggunakan mikroorganisma. Bagi kajian ini, Bionectria sp. telah dipilih bagi bioremediasi minyak motor terpakai. Tempoh inkubasi, peratusan inokulum, peratusan kelembapan tanah, dan peratusan minyak motor terpakai dioptimasikan bagi bioremediasi. Bagi tempoh 6 minggu, Bionectria sp. diinokulasikan untuk menguraikan minyak motor terpakai dari pelbagai peratusan, 3 %, 5 % dan 10 % serta pelbagai peratusan kelembapan tanah, 40 % dan 60 %. Minyak motor terpakai yang tinggal setelah 6 minggu dipencil keluar daripada tanah menggunakan toluene dan ketumpatan optik diperiksa. Keputusan terbaik telah ditunjukkan oleh sampel yang mempunyai 8 % (v/w) inokulum – 60 % kelembapan tanah – 10 % (v/w) minyak motor terpakai dengan 91% minyak terpakai telah diuraikan selepas 6 minggu.

Kata kunci: Bioremediasi, Bionectria sp., minyak motor terpakai, inokulum dan peratus kelembapan

1.0 Introduction

Hazardous pollutants such as Polycyclic Aromatic Hydrocarbon (PAHs) have strained public concerns as PAHs are toxic, mutagenic and also carcinogenic (Bumpus 1989; Clemente et al. 2001; Cerniglia and Sutherland 2001). PAHs are a type of hydrocarbon compound which can be found in the motor oil. Apart from the hazardous characteristics, to make things even worse, during rainy season, flooding or accident could contribute the spread of motor oil from waste pit to the surrounding area and resulting in pollution. Spillage of oil into the environment is a major problem as it may interfere with groundwater source. An efficient technology in term of cost and efficiecny is highly demanded by the world. Bioremediation seem to suit the characteristics of the demanded technology. Bioremediation is a process of using microorganisms to convert hazardous pollutants into less toxic compounds (Odgen and Adams, 1989; Kirch, 2008). This technique involves the manipulation molecular process of compounds degradation through biological activity. Over the past twenty years, fungal bioremediation or mycoremediation become the main desirability for all researchers who involve in bioremediation field.

Fungal decay can be classified into three main types, white-rot, brown-rot and soft-rot (Hudson, 1992). Each type of fungal decay caused by specific species of fungi. White rot fungi which is the common degraders of wood in the nature have the ability to degrade substance that resemble the basic structural unit of lignin including hydrocarbon as both compounds, lignin and hydrocarbon are made of hydrogen and carbon (Pointing, 2001). Furthermore, lignin are built up from phenyl propenoid units and highly hydrophobic which cause them to have the tendency to bioaccumulate in the environment. White rot fungi produce three types of enzymes which involves in the degradation of lignin. The enzymes are lignin peroxidase (LiP), Manganese Peroxidase (MnP), and Laccase (Lac) (Ohkuma, Maeda, Johjima, & Kudo, 2001).

Lately, there are many studies have been done to screen, isolate, characterize fungi which capable of degrading hydrocarbon. The studies mainly carried out to document the potential of fungi in degrading hydrocarbon and thus be applied in bioremediation of hydrocarbon. It would be fascinating to be able to find a fungi species which could degrade a long chain hydrocarbon or repetitive hydrocarbon. Furthermore, fungi have an advantage over bacteria as its own hyphae that capable of penetrating the contaminated soil towards the hydrocarbon as it has spread from the top of the soil (Lysek, 1996).

In order to reveal the full potential of *Bionectria sp.*, optimization of parameters needs to be done before we come up with a complete design of the bioremediation process. Therefore, the objectives of this study is to come up with optimized condition and perform the bioremediation of hydrocarbon contaminated soils by employing the indigenous fungi that capable of degrading the hydrocarbon in oil contaminated soil which is *Bionectria sp.*. The optimized parameters are the time of incubation for the treatment using *Bionectria sp.*, percentage of inoculums used, moisture content of the soil and percentage of the used motor oil applied as contaminant.

2.0 Literature Review

2.1 Hydrocarbon (Used Motor Oil)

Hydrocarbon is an organic compound which consisting entirely of hydrogen and carbon (Solomons & Fryhle, 2008). Majority of hydrocarbons are found in crude oil. Crude oil is a major contaminant of soil and water in oil producing country as a result of extraction and procession of the oil (Ogbo & Okhuoya, 2008). Crude oil will be further processed into motor oil by vacuum distillation. Motor oil constitutes of complex mixture of hydrocarbons and other organic compounds, (Butler & Mason, 1997) that is utilized to lubricate parts of an automobile engine, to smooth the engine operation (Hagwell *et al.*, 1992; Theodori *et al.*, 2003; AMSOIL Inc., 2005). Used motor oil is the brown to black oily liquid removed from the engine of machines when the oil is changed (CEPA, 1994; ATSDR, 1997).

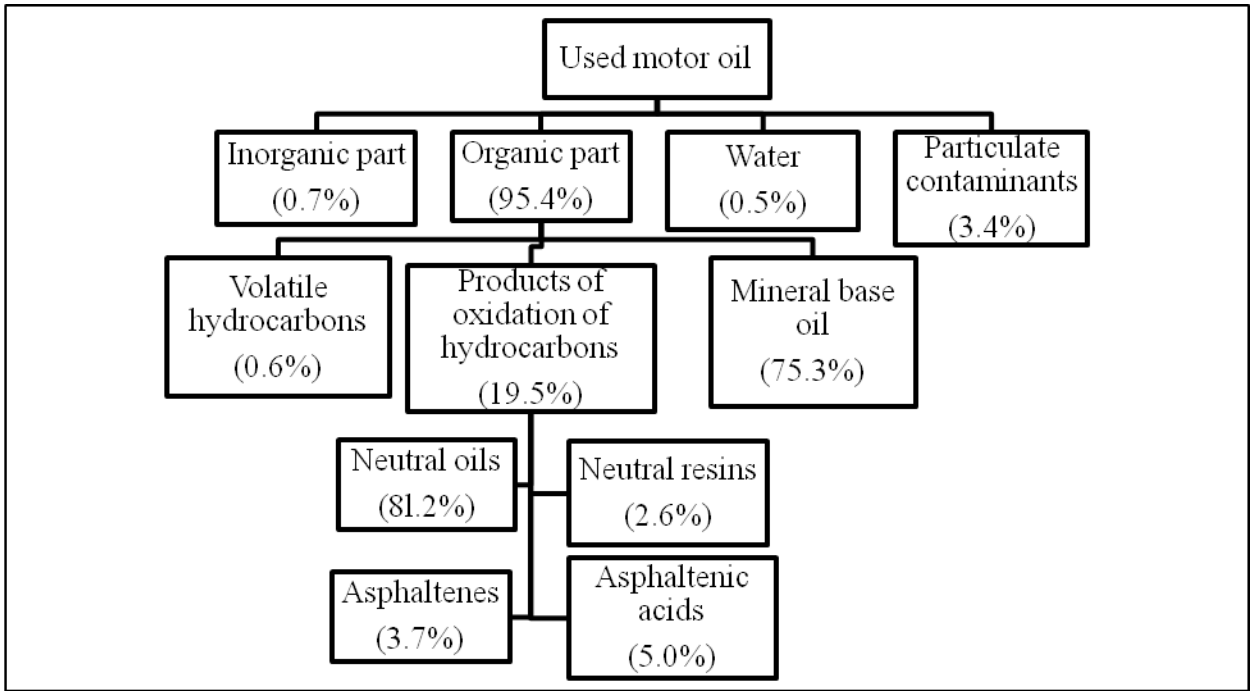

Figure 1. Composition of used motor oil

According to research done by Prokhorenkov, Knyazeva, Ostrikov, and Vigdorovich (2006) as shown in Figure 1, used motor oil have 95.4 % organic parts and 3.4 % particulate contaminants. Out of the 95.4 % of organic part, 75.3 % of them are mineral base oil. This shows that hydrocarbon still present even in used motor oil and proper way of disposing them is needed. Used motor oil contains metals and heavy polycyclic aromatic hydrocarbons (PAHs) that could contribute to chronic hazards including mutagenicity and carcinogenicity (Bumpus, 1989; Clemente *et al.*, 2001; Cerniglia & Sutherland, 2001). Propst *et al.* (1999), Mishra *et al.* (2001), Lloyd and Cackette (2001) reported that prolonged exposure to high used oil concentration may cause the development of liver or kidney disease, possible damage to the bone marrow and also an increased risk of cancer.

In addition to the hydrocarbon base oil and performance-enhancing additives (PEAs) found in fresh motor oil, used motor oil contains chemicals that are formed when oil is exposed to higher temperatures and pressures inside the engines as it works. Investigations and reports claimed that used motor oil generally contain a higher concentration of lead, zinc, calcium, barium and magnesium. Apart from that, it also contain lower concentrations of iron, sodium, copper, aluminium, chromium, manganese, potassium, nickel, tin, silicon, boron, phosphorus and cadmium (Vasquez-Duhalt, 1989; CEPA, 1994, 2002; ATSDR 1997; Irwin *et al.*, 1997; CEPA, 2002). On short-term exposure, the base oil stocks of engine oils are substances of low to moderate toxicity, but studies show that, long-term exposure will cause cancer (WHO 1982; Ridderikhoff 2003).

2.2 Bioremediation using Fungi (Mycoremediation)

Contamination of hazardous compounds such as polycyclic aromatic hydrocarbons (PAHs) in soil, water and air become worldwide apprehension. Due to the magnitude of this problem, rapid, cost-effective and ecological method of decontamination is greatly needed. Bioremediation or mycoremediation may fit these requirements. Bioremediation can be defined as any process that uses microorganisms, fungi or their enzymes to return the contaminated soils to their original condition (Kirch, 2008). Meanwhile, mycoremediation is a process to degrade or remove toxins from the environment by employing fungi only (Stamets, 2005). According to Johnsen *et al*. (2005) biological degradation of PAHs can serve three different functions; assimilative biodegradation that yields carbon and energy for the microorganism itself and at the same time is the mineralization of the compound, intercellular mechanism that render the PAHs water-soluble and hence excreted and cometabolism, which is the degradation of PAHs without production of energy and carbon for the organism metabolism. Cometabolism can be best defined as a non-specific enzymatic reaction with a substance competing with the structurally similar primary substrate for the enzymes active site.

Mycoremediation has attracted interest of researchers in bioremediation which has been focusing on bacteria for a long time. The ability of most fungi to produce extracellular enzymes for the assimilation of complex carbohydrates without prior hydrolysis makes possible the degradation of a wide range of pollutants. They also have the advantage of being relatively easy to grow in fermenters, thus being suited for large scale production. Another advantage is the easy separation of fungal biomass by filtration due to its filamentous structure. Fungal degradation of petroleum hydrocarbon is a very important factor in the treatment of oil pollution both in aquatic and terrestrial environment (Colwell and Walker, 1977; Ibe and Ibe, 1984).

White rot fungi is capable of degrading lignin in nature (Tekere *et al.*, 2001). Lignin is a compound which can be found in plant that is made entirely of hydrogen and carbon element. The same element also made up oil. Scientist manipulates this situation by using fungi that capable of degrading lignin to degrade oil (Hammel, 1997). Fungi are more versatile and more suitable in what concerns to the breadth of substrates they can use. Unlike fungi, bacteria are unable to degrade efficiently polycyclic aromatic hydrocarbons (PAHs) with more than four aromatic rings. The morphology and growth characteristics of fungi are responsible for the rapid colonization of substrates. The branching, filamentous mode of fungal growth allows the fungi to be more efficient in colonization and exploration of the contaminated soil.

Since bioremediation is a microbial process, it requires the provision of several factors or requirements. The first factor that needs to be considered is the concentration of the pollutant that is intended to be degraded. Higher concentration of pollutant may be too toxic for the microorganism and eventually will kill the microorganism. The second factor is the need of specific nutrients which needed by the microorganism in addition to the pollutant as food source. For example, research done by Kaal *et al.* (1993) reported that a few species of white rot fungi are ligninolytic when sufficient nitrogen is present. Nutrient is one factor that can hinder biodegradation if not handled properly and could limit the rate of hydrocarbon degradation in the terrestrial environment (McGill & Nyborg, 1975). Moisture conditions also play a vital role. Fungi are very susceptible to changes in moisture. Therefore, optimized moisture condition is needed to ensure effectiveness of the degradation by fungi.

2.3 Hydrocarbon Degrading Fungi

In fungal wood decay, there are three distinct type of fungal decay can be distinguished: white rot, brown rot and soft rot (Hudson, 1992; Hammel, 1997). White rot fungi are the most abundant degraders of wood in natures (Hammel, 1997). White rot fungi is a physiological grouping of fungi that can degrade lignin and also lignin-like substances. Four main genera of white rot fungi that have shown potential for bioremediation are Phanerochaete, Trametes, Bjerkandera, and Pleurotus (Hestbjerg *et al.,* 2003). These fungi cannot use lignin a source of energy, however, and instead require subtrates such as cellulose or other carbon sources. Thus, carbon sources such as corncobs, straw, and sawdust can be easily used to enhance degradation rates by these organisms at polluted sites. Apart from that, the characteristic of fungi to have mycelia also add to the value of mycoremediation. Filamentous branching of hyphae, forming mycelia allows for more efficient colonization and exploration of contaminated soil by the fungi itself.

Phanerochaete chrysosporium capable of producing extra-cellular enzymes, including lignin peroxidase (LiP) and manganese peroxidase (MnP), which are believed to play role in PAH degradation process (Augustin & Muncnerova, 1994; Bishnoi, Kumar & Bishnoi, 2008). According to research done by Bishnoi, Kumar and Bishnoi (2008), *P. chrysosporium* have the ability to degrade various classes of PAHs in sterilized as well as as in unsterilized soil with given optimum conditions. *P. chrysosporium* also has been claimed to degrade dinitrotoluenes (DNTs), which formed through the transformation of 2,4,6 – trinitrotoluene (TNT) (Bumpus & Tatarko, 1994; Hawari *et al.*, 1999; Hodgson *et al.*, 2000).

Penicillium belongs to the phylum Ascomycota, and various species of Penicillium are abundant in many different environments. Despite of the abundance in the environment, Penicillium in particular have received little attention in bioremediation and biodegradation studies. Additionally, several studies conducted with different strains of imperfecti fungi, *Penicillium spp*. have demonstrated their ability to degrade different xenobiotic compounds such as PAHs with low co-substrate requirements, and could be potentially interesting for the development of economically feasible processes for hazardous pollutant transformation (Scow, Li, Manilal & Alexander, 1990; Husaini, Roslan, Hii & Ang, 2008).

Isikhhuemhen, Anoliefo and Oghale (2008), came up with different method on determining whether there is degradation of crude oil in soil polluted with crude oil. They are utilizing white rot fungus, *Pleurotus tuberregium*. *P. tuberregium* is a basidiomycete's species and can be found in many tropical countries in Africa and South East Asia. From research done by them, *P. tuberregium* is capable of growing on the polluted the soil and transforming the polluted soil into a matrix that is suitable for growth of *Vigna unguiculata*. Twelve days after planting, more than 98% emergence of *V. unguiculata* seeds was observed. Despite of scanty mycelia growth, it did not appear to interfere with germination of *V. unguiculata*.

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2.4 Lignin Degrading Enzymes

Based on previous studies, ligninolysis occurs during secondary metabolism (Hammel, 1997 & Kapachi *et al.,* 1999). White rot fungi degrade lignin by secreting three types of extracellular phenoloxidases; lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) (Ohkuma, Maeda, Johjima, & Kudo, 2001). The ligninolytic enzymes produced by white rot fungi catalyze the degradation of pollutants by using a non-specific free radical mechanism (Pointing, 2001).

According to Glenn *et al.* (1983) and Tien and Kirk (1983), lignin peroxidase (LiP) were the first ligninolytic enzymes to be discovered. LiP is oxidized by hydrogen peroxide (H_2O_2) to a two-electron deficient intermediate, which returns to its resting state by performing two one-electron oxidations of donor substrates (Hammel, 1997). LiP does not only oxidize usual peroxidase substrates, but varieties of non-phenolic lignin structures and other aromatic ethers that resemble the basic structural unit of lignin too (Kresten *et al.,* 1990). Methoxylated benzenes and benzyl alcohols are examples of simplest aromatic substances for LiP and frequently been used by enzymologist to understand theory behind LiP reaction mechanisms.

Manganese peroxidase (MnPs) may be the catalysts that provide low molecular weight oxidants (Glenn *et al.*,1986 & Orth *et al.,* 1993). MnPs can be found produced by most white rot fungi and resemble conventional peroxidases. In addition, Mn(II) is the obligatory electron donor for reduction of the one-electron dificient enzyme to its resting state and producing Mn(III) as a result (Wariishi *et al.,* 1992). Although Mn(III) chelates does not oxidize strongly and are consequently incapable to attack the recalcitrant non-phenolic structures that predominate in

lignin. However, the more reactive phenolic structure that make up approximately 10% of lignin is vulnerable to be oxidize by Mn(III) chelates.

The LiP and MnP catalyzed reactions which described in the last two paragraphs cannot provide the only option on how fungi cleave lignin. Despite of LiP unique properties, is not vital because it is not produced by all white rot fungi during ligninolysis, although most of white rot fungi does produce it. Mn(III) that is generated by MnP cannot be fully accountable because white rot fungi that lack LiP are nevertheless able to degrade the non-phenolic lignin structures that resist attack by chelated Mn(III) (Serebotnik *et al.*, 1994). Therefore another ligninolytic mechanism must be exists. Recent work indicates that the generation of diffusible oxyradicals by MnP may provide the mechanism. MnP promotes the peroxidation of unsaturated lipids, this result in generating transient lipoxyradical intermediates that are believed to act as potent oxidants of other molecules (Moen & Hammel, 1994). The MnP/lipid peroxidation system oxidizes and cleaves non-phenolic lignin model compounds. It also depolymerizes both non-phenolic and phenolic synthetic lignin (Bao *et al.*, 1994).

Laccases (Lac) are blue copper oxidases that catalyze the one-electron oxidation of phenolics and other electron-rich substates (Hammel, 1997). Most ligninolytic fungi are capable of producing Lac, however, *P. chysosporium* is an exception from the list. Lac has multiple copper atoms which will be reduced as the substrates are oxidized. After receiving four electrons by laccase moleucle, lac reduces molecular oxygen into water, returning it to the native state. The action of lac on lignin is similar to Mn(III) chelates as the phenolic units are oxidized to phenoxy radicals, eventually lead to degradation of some structures (Kawai *et al.*, 1988).

3.0 Materials and Methods

3.1.0 Materials preparation

One thousand gram of sand were obtained from sand stock which available in the Genetic Molecular Laboratory, UNIMAS. Prior to use, sand were allowed to air-dried and then filtered using a sieve with opening of 710 micrometer.

Soil was collected using slight modification of method proposed by Bishnoi, Kumar and Bishnoi, (2008). Five thousand gram of soil was collected from a single location in UNIMAS, Kota Samarahan. Soil was then stored in sterile plastic bag at room temperature. Prior to use, soil was allowed to air-dried and then sieved with sieve of 710 micrometer opening to remove debris.

3.2.0 Microbial preparation

Four hundred ml of Potato Dextrose Agar (PDA) (OXOID) was prepared and poured into plastic Petri dish. *Bionectria sp*. was obtained from UNIMAS Microbial Collection and cultured on the agar for five days at 37°C to be use as working culture. After five days, 1cm X 1cm block of PDA with fungi grown on it was cutted and transferred into a new PDA to be cultured at 37°C for stock culture. One hundred ml of Malt Extract (ME) (Merck) broth was prepared in a 250 ml conical flask and sterilized. One cm X one cm block of PDA with fungi grown on it was cutted from working culture. The block was then transferred into the 100 ml ME. The flask was shaken on orbital shaker for one week at room temperature before used in the experiment.

3.3.0 Standard Curve Preparation

Standard curve for volume of used motor oil against absorbance at 420 nm was prepared by diluting different volume of used motor oil in 15 ml of toluene. One thousand µl, 500 µl, 250 µl, and 125 µl are diluted in separate beaker with 15 ml of toluene in each beaker. Each mixture was filtered and the filtered solution is placed inside a glass cuvette and the absorbance reading was obtained using spectrophotometer (BioChrom Libra S12). This process was repeated twice so that average reading is obtained.

3.4.0 Motor oil degradation experiment

Bioremediation by utilizing fungi requires optimization of several parameters including time of incubation, percentage of inoculum used and percentage of the target pollutant itself in order to make the process more effective. Therefore, different parameters which determine the effectiveness of the degradation process were optimized as described below.

3.4.1 Time of incubation

The optimization process of time of incubation was carried out based on method proposed by Ijanh and Antai, (2003). Two hundred gram of sand was placed in 500 ml beaker. Moisture content of the sand in the 500 ml beaker was fixed to 50 % by adding distilled water and allowed to settle overnight. Ten gram of sand was transferred from the beaker into 15 plastic Petri dish. One percent (v/w) of used motor oil was added into all 15 plastic Petri dish. The sand and used motor oil is mixed by using spatula and allowed to settle overnight. Next two gram of sawdust was then added into the mixture of sand and used motor oil.

Finally, one percent (v/w) of *Bionectria sp*. which grown in ME is inoculated in 10 of the 15 plastic Petri dish as the remaining five plastic Petri dish act as control, without fungi inoculated inside it. The mixture was again mixed before left for incubation at room temperature. The incubation period was limited to five weeks, where every week, three plastic Petri dish was analyzed for the degraded used motor oil. The three plastic Petri dish represent duplicate of sample, and a control.

Two hundred gram of soil is placed in 500 ml beaker. Moisture of the soil in the 500 ml beaker is fixed to 50 % by adding distilled water and allowed to settle overnight. Ten gram of soil is transferred from the beaker into 18 plastic Petri dish. One percent (v/w) of used motor oil was added into all 15 plastic Petri dish. The soil and used motor oil was mixed by using spatula and allowed to settle overnight. Next two gram of sawdust was then added into the mixture of soil and used motor oil.

Finally one percent (v/w) of *Bionectria sp*. which grown in ME broth is inoculated in 12 of the 18 plastic Petri dish as the remaining six plastic Petri dish will be control, without fungi inoculated inside it. The mixture is again mixed before left for incubation at room temperature. The incubation period is limited to six weeks, where every week three plastic Petri dish was analyzed for the used motor oil present. Three plastic Petri dish represent duplicate of sample, and a control.

3.4.2 Percentage of inoculum

Volume of inoculum interferes with biodegradation rate as low volume of inoculums will retard the biodegradation process. Therefore, percentage of inoculums was tested based on method used by Wolski, Murialdo and Gonzalez (2006) with modification from the original method. Four percent, six percent, and eight percent (v/w) *Bionectria sp*. cultured in ME was inoculated in different plastic Petri dish which contain ten gram of dried soil, two gram of sawdust, and one percent (v/w) oil. For the soil moisture content, it was fixed to 50 %. Time of incubation was set to six weeks based on the best time of incubation for degradation from the previous experiment. For the control, no incoulum of *Binocteria sp.* was included in the plastic petri dish. This experiment was designed in duplicate of sample and a control. Every week, three plastic Petri dish was analyzed for remaining of used motor oil present.

3.4.3 Moisture of the soil

Fungi growth is susceptible to changes is moisture content of its environment. Is is vital to determine what moisture content a fungi favor the most. Ten gram of the air-dried soil was moisten to different moisture, which are 40 %, and 60 % (Trenk & Hartman, 1970). The ten gram of moisten soil was placed in different plates which contain two gram of sawdust, and one percent (v/w) oil, and the best percentage of inoculums was incubated for a period of six weeks. For the control, no incoulum of *Binocteria sp.* was included in the plastic petri dish. This experiment is set up in duplicate of sample and a control. Every week, three plastic petri dish were analyzed for remaining of used motor oil present.

3.4.4 Percentage of oil used

Higher concentration of pollutants may be too toxic to be utilized by fungi. Proportion of pollutant and percentage of inoculum need to be determined in order to have the suitable ratio of these two factors. Three percentages of oil based on volume over weight (v/w), which is three percent, five percent, and ten percent added into different plates together with ten gram of soil, two gram of sawdust, the best percentage of inoculums and preferred moisture for the soil based on previous experiment. Time of incubation was limited to six weeks. For the control, no incoulum of *Binocteria sp.* was included in the plastic Petri dish. This experiment is set up in duplicate of sample and a control. Every week, three plastic Petri dish was analyzed for remaining of used motor oil present.

3.4.6 Hydrocarbon analysis

Quantitative determination of motor oil extraction is carried out using modified methods of Udeme and Antai (1988) and Hadibarata and Tachibana (2009). Each of the ten gram of soil samples were placed in a 50 ml flask and mixed with 15 ml of toluene (AnaLar grade). The flask was then shaken vigorously for five minutes. The liquid phase was separated by allowing it to pass gradualy through a funnel fitted with filter paper (no. 5A, 110 mm). Subsequently, the filtered liquid phase was collected using a 50 ml beaker. The filtered liquid was transferred into glass cuvette and then checked for their optical density at wavelength of 420 nm using spectrophotometer (BioChrom Libra S12).