STUDY ON BIODEGRADATION OF MIRI AND MASILA CRUDE OIL AND USED CAR OIL BY MICROORGANISMS ISOLATED FROM MALAYSIAN SOIL AND THE EFFECT OF AERATION AND NPK ADDITION ON BIODEGRADATION PROCESS

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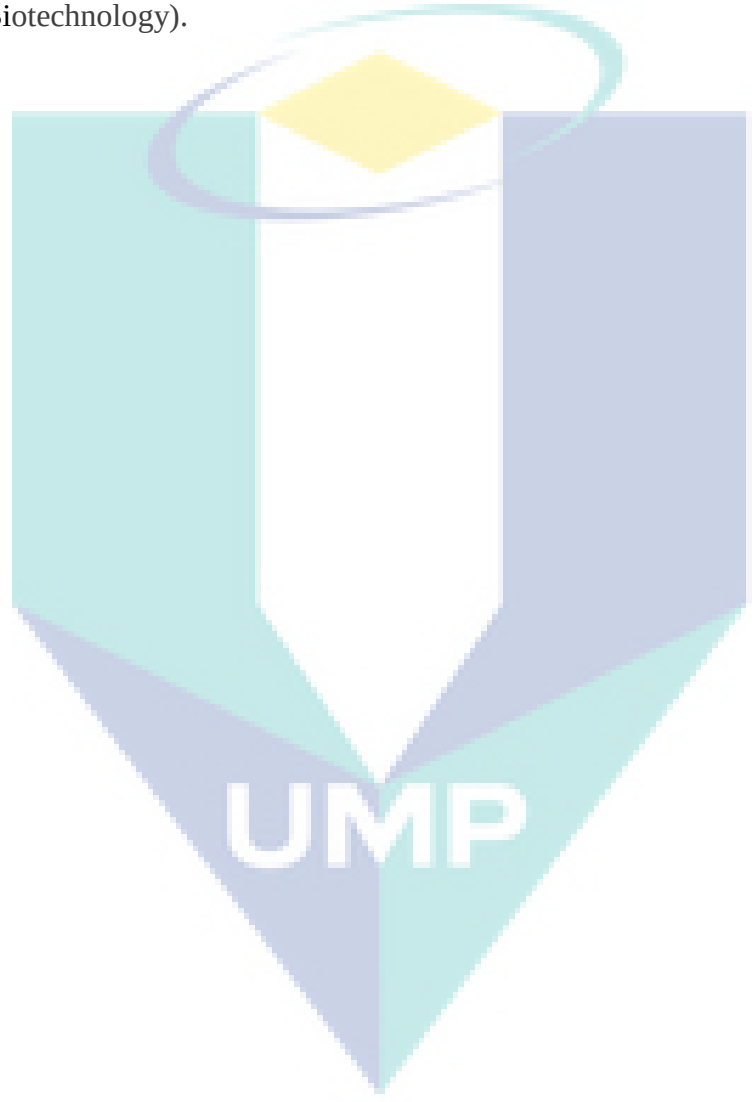

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ABSTRACT

In this study, five contaminated soil samples with benzene, used car oil and diesel were collected from five car workshops in Kuala Lumpur area, Malaysia. The microbial strains were isolated using selective media (agar containing crude oil, used car oil and benzene). Microorganisms were identified by biochemical test and then used in biodegradation experiment of two types of crude oils (Miri and Masila) and used car oil. Qualitative determination of the degradation capacity of crude oils and used car oil was driven in 24 well cell culture cluster - flat bottom, adding to each well nutrient broth medium, crude oil or used car oil and the isolated microorganism cultured in the tubes (single and mixtures) and incubated at 27-°C for 30 days. For hydrocarbons rate of biodegradation measurement, sterilized soil was distributed in petri dishes, 3% w/w of two crude oils and used car oil were added, separately and then supplemented with isolated strains (single and mixtures). One Petri dish was used as a control without any microbial addition. Absorbance was determined by spectrophotometer at 360 nm and at 340 nm for crude oils and used car oil, respectively. The effect of aeration, added NPK and added microbial degraders on biodegradation of Masila crude oil and used car oil was studied. Soil was supplemented with 20% Masila crude oil and used car oil, separately. The soil was distributed into containers. Container (1) was aerated twice a week, NPK 1:1:1 was added to container (2), mixture of all isolated strains with degrading capacity was added to container (3). Finally, NPK with continued aeration in addition to the microbial mixture were applied to the last container (4). One container was used as a control without any addition (soil and contaminant only). Colony Forming Unit (CFU) of total heterotrophic microbes and hydrocarbon utilizing microbes, PH and percentage of oils degradation were determined. 22 microbial strains were isolated and identified as, *Achrombacter*, *Aeromonas*, *Klebsiella pneumonia*, *Pseudomonas*, *Corynebacterium*, *Penicillium*, *Enterobacteriaceae* (*Enteric rods*), *Actinobacillus*, *Streptomyces*, budding yeast cells, *Cladosporum* and *Geotrichum* spp. The highest biodegradation result in Miri crude oil after 30 days were 54.33% and 84.61% for strain Z13 (*Corynebacterium* spp.) and microbial mixture of the strains isolated from Rawang and Serdang area (MS), respectively. While in Masila crude oil was 33.81% for Strain A3 (*Klebsiella pneumonia*) and 49.47% for microbial mixture of the strains isolated from Serdang (Smix). In used car oil biodegradation experiment, strain Z4 (*Corynebacterium*) had the highest degradation with 72.9%. While microbial mixture of the strains isolated from Kajang (Zmix) had 72.4 % of degradation. In the experiment of the effect of aeration, added NPK and added microbial degraders on biodegradation, the aerated container showed 56.62% of degradation after 42 days in Masila crude oil, while the container which contained NPK and was aerated and supplemented with isolated strains showed 66% degradation in used car oil. Generally, two bacterial species and one fungal species isolated were found to be effective degraders (*Corynebacterium* spp, *Streptomyces* spp. and *Cladosporum* spp,), respectively. The highest degradability by single strains was on used car oil which might be due to the adaptability of the isolated microbes to use it. The microbial mixtures showed higher effect on the biodegradation than the single strains. Aeration found to be the most important in the effect on the biodegradation results.

ABSTRAK

Sebanyak Lima sampel tanah tercemar dengan benzene, minyak kereta terpakai dan diesel diambil daripada lima bengkel kereta di sekitar Kuala Lumpur, Malaysia untuk tujuan kajian ini. Bagi tujuan itu, strain mikrob diasingkan dengan menggunakan medium terpilih (agar yang mengandungi minyak mentah, minyak terpakai kereta dan benzene).Ujian biokimia juga dijalankan untuk mengenalpasti mikroorganisma yang ada dan digunakan dalam eksperimen biodegradasi dua jenis minyak mentah (Miri dan Masila) dan minyak terpakai kereta. Penentuan secara kualitatif kapasiti degradasi minyak mentah dan minyak terpakai kereta dijalankan dalam 24 bekas kluster kultur sel – yang mempunyai dasar yang rata. Brot nutrient medium , minyak mentah dan minyak terpakai kereta dimasukkan ke dalam setiap bekas. Kultur mikroorganisma ini kemudiannya diasingkan dalam tiub (tunggal dan bercampur) dan diinkubasi pada suhu 27°C selama 30 hari. Bagi mengukur kadar hidrokarbon biodegradasi, tanah yang telah disteril dimasukkan kedalam piring petri dan ditambah dengan 3% w/w minyak mentah dan minyak terpakai kereta secara berasingan dan kemudiannya ditambah dengan strain yang diasing (tunggal dan bercampur). Salah satu daripada piring petri tersebut digunakan sebagai piring kawalan tanpa menambah apa-apa mikrob. Daya serap minyak mentah ditentukan dengan spektrofotometer pada 360nm dan untuk minyak terpakai kereta pada 340nm. Kajian juga dibuat untuk melihat kesan pengudaraan, penambahan NPK dan penambahan mikrob pengdegradasi pada biodegradasi minyak mentah Masila dan minyak terpakai kereta. Tanah tersebut telah ditambah dengan 20% minyak mentah Masila dan minyak terpakai kereta secara berasingan.Tanah tersebut kemudian dibahagikan kepada beberapa bekas. Bekas (1) diudarakan dua kali seminggu, NPK bernisbah 1:1:1 pula dimasukkan kedalam bekas (2), kesemua campuran strain yang diasingkan dan mempunyai kapasiti degradasi dimasukkan kedalam bekas (3). Disamping campuran mikrob, NPK yang diudarakan secara berterusan dimasukkan kedalam bekas (4). Satu bekas pula digunakan sebagai bekas kawalan tanpa menambah bahan lain(tanah dan bahan cemar sahaja).Unit Pembentukan Koloni (Colony Forming Unit) keseluruhan mikrob heterotropik dan mikrob menggunakan hidrokarbon, PH dan peratus degradasi minyak juga ditentukan.Sebanyak 22 strain mikrob diasingkan dan dikenalpasti sebagai *Achrombacter, Aeromonas, Klebsiella pneumonia, pseudomonas, Corynebacterium, Penicillium, Enterobacteriaceae (Enteric rods), Actinobacillus, Streptomyces,* budding yeast cells, *Cladosporum dan Geotrichnum* spp. Selepas 30 hari keputusan biodegradasi tertinggi bagi minyak mentah Miri ialah 54.33% dan 84.61% untuk strain Z13 (*corynebacterium* spp*)* dan strain campuran mikrob yang diasingkan dari Rawang dan Serdang secara berturut. Sementara itu peratusan untuk strain A3 (*Klebsiella pneumonia*) untuk minyak mentah Masila ialah 33.81% dan 49.47% untuk strain mikrob campuran yang diasingkan dari Serdang (Smix).Dalam eksperimen biodegradasi minyak kereta, strain Z4 (*Corynebacterium*) menunjukkan peratus degradasi tertinggi iaitu 72.9%. Sementara itu campuran mikrob strain yang diasingkan dari Kajang (Zmix) menunjukkan 72.4% degradasi. Dalam eksperimen yang mengkaji kesan pengudaraan dan penambahan NPK keatas biodegradasi menunjukkan bahawa bekas yang diudarakan mengalami degradasi sebanyak 56.62% selepas 42 hari di dalam minyak mentah Masila. Sementara itu bekas yang mengandungi NPK yang diudarakan dan ditambah dengan strain yang diasingkan menunjukkan degradasi sebanyak 66% dalam minyak kereta. Secara amnya, dua spesis bakteria dan satu spesis fungus terasing didapati berkesan sebagai pengdegradasi (*Corynebacteruim* spp, *Streptomyces* spp. Dan *Cladosporum* spp). Degradasi tertinggi oleh strain tunggal ialah pada minyak terpakai kereta mungkin disebabkan oleh kebolehan mengadaptasi mikrob terasing yang digunakan. Campuran mikrob menunjukkan kesan yang lebih tinggi keatas biodegradasi berbanding strain tunggal. Pengudaraan merupakan sesuatu yang penting dalam memberi kesan kepada keputusan biodegradasi.

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Ca	Calcium
CO_2	Carbon dioxide
°C	Celsius
cm	Centimeter
g	Gram
hr	Hour
Kg	Kilogram
Km	Kilometer
Pb	Lead
L	Liter
<	Lower than
Mg	Magnesium
m	Meter
µg	Microgram
µL	Microliter
µL/L	Microliter per liter
µm	Micrometer
mg	Milligram
mg/L	Milligram per liter
mL	Milliliter
mm	Millimeter
min	Minute
nm	Nanometer

ppm	Part per million
%	Percentage
pH	Potential hydrogen
sec	Second
v/v	Volume per volume
w/w	Weight per weight
Zn	Zinc

LIST OF ABBREVIATIONS

NH_4NO_3	Ammonium nitrate
$(NH_4)_2SO_4$	Ammonium sulfate
ANOVA	Analysis of variance
BTEX	Benzene, toluene, ethylbenzene, and the xylenes
C	Carbon
CA	Constant aeration
DNA	Deoxyribonucleic acid
Na_2HPO_4	Disodium hydrogen phosphate
dH_2O	Distilled water
FID	Flame ionization detector
GC	Gas chromatography
GCms	Gas chromatography-mass spectrometry
G -ve	Gram negative
G +ve	Gram positive
HMW	High molecular weight
HMW PAH	High molecular weight polycyclic aromatic hydrocarbons
IA	Intermittent aeration
$MgSO_4$	Magnesium sulfate
MSB	Minimal salts basal
mix	Mixture
NPK	Nitrogen, phosphate and potassium
N/P	Nitrogen phosphate ratio
NAPL	Nonaqueous phase liquid

PHCs	Petroleum hydrocarbon contents
PCBs	Polychlorinated biphenyls
PAHs	Polycyclic aromatic hydrocarbons
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
rRNA	Ribosomal ribonucleic acid
NaCl	Sodium chloride
SDS	Sodium dodecyl sulphate
Spp.	Species
SD	Standard deviation
SPSS	Statistically backage for social sciences
TPH	Total petroleum hydrocarbon
TCE	Trichloroethylene
UV	Ultraviolet
UK	United kingdom
US	United states
USA	United states of America
UKM	Universiti kebangsaan malaysia
UMP	Universiti Malaysia Pahang
UPM	Universiti Putra Malaysia
WA	Without aeration

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

The quality of life on the planet depends on the quality of the environment. Years ago, we were sure that we had many land and resources; today, however, the real picture shows, in greater or lesser degree, our negligence and carelessness in using them. In many countries the problems associated with contaminated sites became more serious. Contaminated sites in general resulted from previous industrial activities, when the connection between production, use, and disposal of hazardous substances with health and environmental effects were less well recognized than today. The problem is global, and the estimated number of contaminated sites is significant (Cairney, 1993). The awareness of potential threat to human health because of contaminated land is now much bigger, and its continual discovery over recent years has led to international efforts to remedy many of these sites, either as a response to the risk of adverse health or environmental effects caused by contamination or to enable the site to be redeveloped for use. (Vidali, 2001).

Fossil fuels represent primary energy source in the global industry. Due to fossil fuel manufacturing and imports/exports, there is a big threat on environmental pollution, and serious ecological damage. This is the result of fuel by-products and spills in sites where storage, transport, distribution, refining, consumption and the existing industries related fossil fuel can cause harm. The interest of scientists to investigate the oil distribution and its fate in the environment increases because of this fact, especially in the marine environment. About five million tons of crude oil and refined oil go into the ecosystem every year due to anthropogenic sources from oil spills (Hinchee and Kitte,

1995). Analysis of reported oil spills shows that most of the oil comes from tankers, barges and other vessels as well from land pipeline spills. Extensive changes in marine, in addition to terrestrial ecosystems resulting from the grounding of the Exxon Valdez (1989), the Nahodka oil spill, the Erica spill (1999) and the Prestige spill (2002), have increased the attention of biotechnologists, chemists, environmentalists, and engineers (Braddock et al., 1995; Tazaki et al., 2004).

Used engine oil is one of the sources for soil pollution with hydrocarbons. Used engine oil contains petroleum ether, benzene, gasoline, naphthalene, mineral spirits, kerosene, and fuel oil, paraffin wax, lubricating oil, tar or asphalt. Used car oil has much higher concentrations of PAHs (polycyclic aromatic hydrocarbons) compared with new motor oil (Irwin et al., 1997).

Traditional remediation methods include containment material and physical removal. These methods also use chemicals, especially shoreline cleaners, which are usually organic solvents with or without surfactants (Riser-Roberts, 1992). The cleaning of shoreline with surfactants emulsifies the adsorbed oil, which can entrain adjacent waters or is even transported deeper into the soil of the shoreline. The oil solvent mixtures are collected by conventional skimming methods. Mechanical recovery of oil includes oil sorbents. Sorbents can transform oil to a transportable form for short-term storage. Almost all the physicochemical methods use chemical agents, in addition to their emulsion with oil, which can cause toxicity, to aquatic organisms. They represent another source of pollution and increase the cost of the oil recovery. Additionally, abiotic losses because of evaporation of low molecular hydrocarbons, dispersion and photooxidation (for aromatic compounds only) play an important role in decontamination of the oil spill environments (Mills et al., 2003).

There is an increased interest to promote environmental methods in the process of cleaning oil-polluted sites. These methods cost less and do not introduce chemicals to the environment. In comparison with physiochemical methods, bioremediation is a very feasible alternative for an oil spill response. This technique is considered an effective technology for treatment of oil pollution. One reason is that the majority of the molecules in the crude oil and refined products are biodegradable (Malatova, 2005).

1.2 PROBLEM STATEMENT

Because of the rapid industrialization processes and the big worldwide energy demands, oil spills become a global problem, especially in industrialized countries. International Oil Spill Database shows that since the early 1960s, nearly 1135 million litres of oil have spilled into US marine waters which occurred in 826 incidents involving tankers, barges, and other vessels, and about 757 million litres of oil have spilled onto US soil from the land pipeline spills (on average, 99 land pipeline spills per year). An estimated 1892 million and over 757 million litres of oil have spilled from tankers in Europe and Asia Pacific since 1965, respectively. The high quantities of spilled oils and petroleum products go into our sea, territory as well as groundwater, which cause severe damage to marine life, terrestrial life, human health, and natural resources (Wang et al., 1999). One example of the most recent oil spill accident is the Deep water Horizon oil spill or the BP oil spill in the Gulf of Mexico which happened in 2010. It is the largest accidental marine oil spill in the history of the petroleum industry. About 4.9 million barrels of crude oil was released into the sea (Rick and Alan, 2010).

In Asia during the last few years, there were major oil spill accidents at different places of the country in January 21/1993 in Singapore, Indonesia, and Malaysia**.** Two million barrels of oil was leak and burned, and the leak spread a slick approximately 35 miles (56 km) along Sumatra drifting towards India's Nicobar Islands. In September 2/2000 Malaysia, 116 Tons of diesel oil spilled after a cargo sank due to a collision. In May 28/2001 Malaysia, an oil tanker with 67 tons of diesel fuel, and 1,500 Tones of bitumen, sank after a crash from behind by a super tanker. Diesel and bitumen started to spill into the sea, and spread to about one nautical mile from the spot of collision. In June 13/2001 Malaysia, an Indonesian tanker loaded with a toxic chemical capsized off Malaysia's southern state of Johor, 18 Tons of diesel, and 600 Tons of the poisonous industrial chemical phenol were spilled. The toxic spill killed thousands of marine creatures, and Singapore authorities have also warned its citizens to stay away from nearby waters (The Marine Group).

Engine oil is the most worldwide used petroleum product. The inevitable leakage and abandoned remnant of engine oil that is used as lubricant pollute the environment

(Liang et al., 2007). Only 1 gallon of used motor oil is enough to contaminate 1000 000 gallons of freshwater. Used engine oil also represents potential threat to humans, animals, and vegetation (Edewor et al., 2004). The most significant and largest proportion of oil spill is from internal combustion engines (petrol and diesel engines) in road transport and shipping (Porst, 2000). The components in used motor oil cause harmful effects on liver, kidneys, heart, lungs and nervous system (Irwin et al., 1997). One of the most significant impacts associated with workshop seepage of used motor oil includes loss of soil fertility, holding capacity of water, permeability and binding capacity (Khan and Rizvi, 2011).

Interest in the microbial biodegradation of pollutants was intensified recently to find sustainable ways to remediate contaminated environments. Bioremediation and biotransformation are methods, which use the naturally occurring ability of microbial metabolism to degrade, transform or accumulate a huge range of compounds including hydrocarbons such as crude oil and its different products (Alexander, 1999).

1.3 OBJECTIVES

The primary objectives of this study are:

(i) To isolate and identify hydrocarbon degrading microorganisms from contaminated soil in Malaysia by selective enrichment techniques.

(ii) To determine the rate of biodegradation of two types of crude oil (Miri and Masila) and used car oil by isolated microorganisms

(iii) To study the ability of single strains in comparison with microbial blends to degrade petroleum and used car oil.

(iv) To study the effect of aeration, NPK and microbial degraders, which have been added to Masila crude oil and used car oil biodegradation.

1.4 SCOPES OF STUDY

- Collection of contaminated soil samples from various car workshop areas in Kuala Lumpur region (Kuala Lumpur, Selangor, Kajang, Rawang and Serdang), which are known to be contaminated with different types of hydrocarbons such as benzene and used engine oil.

- Microorganism's isolation by using selective mediums (agars containing crude oil, used car oil and benzene).

- Identification of isolated microorganisms by using simple Gram staining technique and biochemical tests to determine the type of the strain which was effective as a hydrocarbon degrader.

- Determination of the rate of biodegradation of three types of hydrocarbons (used car oil, Miri and Masila crude oil) quantitatively, using spectroscopic techniques to determine microbial efficacy as hydrocarbon degrader.

- Finally, study of the effect of aeration, NPK and microbial degraders adding on used car oil and Masila crude oil biodegradation using spectroscopic technique.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Contamination of soil and water with hydrocarbons poses a major ecological and human health problem that needs an effective and affordable technological intervention. Many sites stay contaminated with no treatment in sight because it is very expensive to clean them with the available technologies. Bioremediation can provide an economic solution for remediating many of these sites (Martello, 1991).

Bioremediation is the application of biological treatment to the cleanup of harmful chemicals. This process includes detoxification, where the waste is converted to less toxic compounds, and mineralization, which is a process of transforming the waste materials into inorganic compounds such as carbon dioxide, water and methane (Gavrilescu, 2010; Martello, 1991). Bioremediation is one environmental biotechnology technique. For many years, microorganisms have been used to eliminate organic matter and toxic chemicals from domestic and industrial waste effluents. However, the range of treatments has been developed greatly due to the great improvement in available biotechnology. Bioremediation is now the technology of choice for the remediation of many polluted environments, especially areas contaminated with petroleum hydrocarbons. Recently, serious efforts have been made to use nature's biodegradative ability aiming at large-scale technological applications for effective and economic environmental restoration (MABIC, 2005).

The regular techniques used for remediation depend on digging up contaminated soil and removing it to a landfill, or capping and containing the contaminated areas of a

site. The methods have some disadvantages. The first method simply transfers the contamination somewhere else and may make major risks in the drilling, handling, and transporting of hazardous material. Moreover, it is very difficult and expensive to find new landfill sites for disposal of the material. The cap and contain method is only a temporary solution because the contamination remains on site, requiring observation and maintenance of the isolation barriers long into the future, with all the associated costs and potential responsibility (Vidali, 2001).

A better way than these conventional methods is to completely destroy the contaminants if possible, or transform them to less harmful substances. High-temperature incineration and various types of chemical decomposition (e.g., base-catalyzed dechlorination, UV oxidation) are the technologies that have been used. They can be effective at minimizing levels of any contaminants, but they have several side effects. Generally, the technology is very complex; the cost for small-scale application is high, and the difficulty of being accepted by public, particularly for incineration, which can increase the exposure to contaminants for workers at the site and residents living nearby (Vidali, 2001).

Bioremediation offers the possibility of destroying or rendering harmless various contaminants by using naturally occurring biological activity. It uses relatively cheap, simple technology techniques, which mostly have a high public acceptance and may often be carried out on site. It cannot be suitable all the time;, however, if the range of pollutions on which it is effective is limited, the time scales involved are prolonged, and the levels of residual contaminant that can be achieved may not always be suitable. Though the methods employed are not complex, large experience and expertise will be needed to design and apply a good bioremediation program, because of the need to thoroughly assess a site for suitability and to optimize conditions to fulfill a satisfactory result (Vidali, 2001).

Bioremediation has been used at many sites over the world, including Europe, with different degrees of achievement. Techniques are being developed as more experience and knowledge are gained, and bioremediation has great potential for treating certain types of site contamination. Unfortunately, the principles, techniques,

advantages, and disadvantages of bioremediation are not widely known or understood, especially among those who will have to deal directly with bioremediation proposals, such as site owners and regulators. Here, we intend to assist by providing a straightforward, pragmatic view of the processes involved in bioremediation, the pros and cons of the technique, and the issues to be considered when dealing with a proposal for bioremediation. Some tests make an exhaustive examination of the literature of bioremediation of organic (Norris et al., 1993) and inorganic pollutants (Hinchee and Kitte, 1995), and another test takes a look at pertinent field application case histories (Flathman et al., 1993).

Polycyclic aromatic hydrocarbons (PAHs) are very important pollutants found in soil, air and sediments. These contaminants are introduced into the environment through various methods. PAHs products and their derivatives are widely distributed due to incomplete combustion of organic substances arising, in apart from natural combustion as in volcanic eruptions and forest fires, but the most important reason is human intervention such as industries and the related accidents. In recent years, industrial production, transportation, storage, refining, consumption and distribution are the major causes of PAHs contamination. The fate of polycyclic aromatic hydrocarbons in environment is of great concern due to their toxic, mutagenic, and carcinogenic effects (Fawell and Hunt, 1988; Mas et al., 2010). PAHs can permeate into organic-rich soils and sediments, can be accumulated in marine living creatures, and then transferred to humans through seafood consumption (Meador et al., 1995).

2.2 BIOREMEDATION

As a definition, bioremediation is the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms. It uses natural bacteria and fungi or plants to degrade or detoxify substances harmful to health of human and/or the environment (De Wilde et al., 2007; Vidali, 2001). By other means, bioremediation is the intentional use of biological degradation procedures to remove or reduce the concentration of environmental pollutants from sites where they have been released. The concentrations of pollutants are reduced to levels considered acceptable to site owners and/or regulatory agencies (Figure 2.1). The microorganisms

could be indigenous to contaminated sites or they may be isolated from somewhere else and introduced to the contaminated area. Pollutant compounds are transformed by organisms through reactions that happen as part of the metabolic processes. Compound biodegradation is normally a result of the actions of a group of organisms (Vidali, 2001).

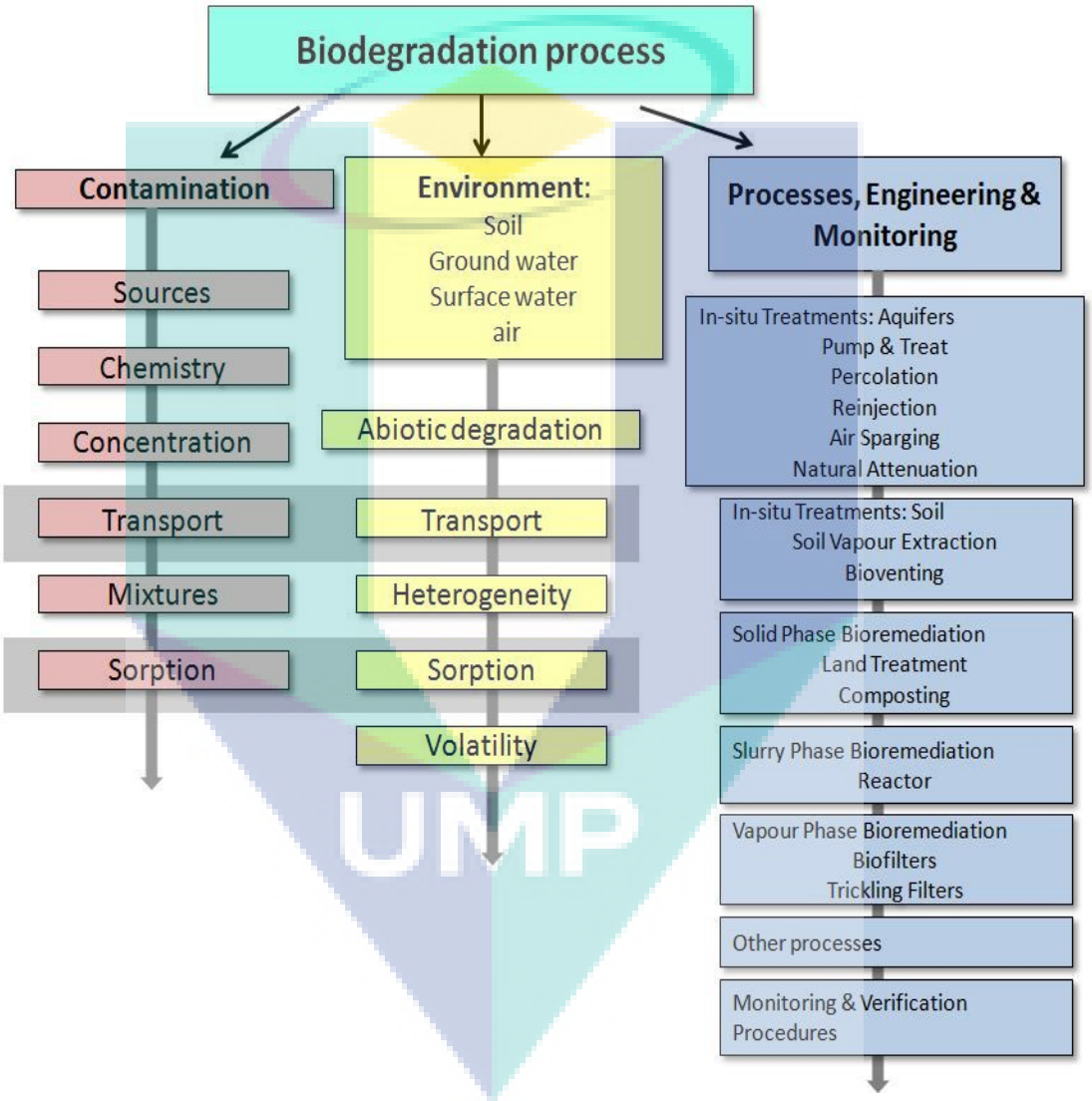


Figure 2.1 Overview of Bioremediation Process

Source: Santra (2010)

2.2.1 History of Bioremediation

Bioremediation is not a new concept;, scientists have discovered the process since 1940s. Bioremediation was made known to the public in the U.S. only in 1980s as a procedure of removing contaminants from coastal area polluted with oil. The *Exxon Valdez* oil spill in 1989 in Prince William Sound, Alaska was the first step for this concern. Since 1989, bioremediation has been involved, discussed, and applied in many circumstances (Hoff, 1993).

The bioremediation evolution as an oil spill removing technology gives a good example of how a new ecological technology evolves. The history of bioremediation in spill response is divided into three periods (Hoff, 1993);, 'courtship' period, which is prior to 1989, the 'honeymoon' period, which is from 1989 until 1991 and the 'establishment' period, which is after 1992. The courtship period (Pre-1989) was a research period, when bioremediation was not well known outside the microbiology or hazardous waste community. Numerous published articles from 1970s and earlier reported the process of microbial degradation of oil, both in the laboratory and in field experiments. Some scientific papers on this issue were published during the 1970s and 1980s, as well as many review papers covering mechanisms of biodegradation, and papers submitting results from controlled field trials measuring rate of degradation in different environments. Many studies, which followed large oil spills like the *Amoco Cadiz*, measured degradation of oil in the environment and confirmed earlier published results from laboratory studies (Hoff, 1993). During the honeymoon period (1989 to 1991), bioremediation broadened attention and interest. The end of this period was disappointing as the promise of the technology was not always fruitful by its application in real field. This period ended in 1990 and 1991, and as the result, monitoring of the bioremediation applications became available. None of the studies conducted outside Alaska could confirm the effectiveness of bioremediation applications in field tests. Many of these tests had poor design, were conducted over very short a time period, or had analytical difficulties measuring changes in oil concentrations (Hoff, 1993). The establishment period started in 1992. During this period, bioremediation has achieved a certain level of acceptance, with more realistic expectations than before, but the interest and attention level has decreased considerably. The doubt about the toxicity of several

fertilizer preparations and microbial products, and questions about their effectiveness decreased wider use of bioremediation on marine coastlines. Most proposals to use bioremediation in open coastal environments are now joined by some types of monitoring program to evaluate whether the technique can enhance oil degradation above background rates. In the situation of a major spill, such as the *Exxon Valdez*, a pilot test can be conducted where the responsible authorities agree to the use of bioremediation on a large scale. However, the coast and effort required to establish a monitoring program may deter the use of bioremediation at smaller spills (Hoff, 1993).

2.2.2 Principle of Bioremediation

Bioremediation is the process where organic wastes are degraded biologically under controlled conditions to levels below concentration limits established by regulatory authorities (Kumar et al., 2010; Mueller et al., 1996).

For bioremediation to be efficient, microorganisms must attack the pollutants enzymatically and convert them to harmless metabolites. Bioremediation can be effective only where environmental conditions allow microbial growth and activity. Its application involves the manipulation of environmental parameters to permit microbial growth and degradation to take place at a faster rate. There are no rules to predict how the contaminant can be degraded. Bioremediation are typically more economical than other traditional methods such as incineration, and some contaminants can be treated on site to reduce exposure risks for clean-up personnel, or wider exposure through transportation accidents. Since bioremediation basically depends on natural attenuation, the public considers it more acceptable than other technologies (Vidali, 2001). Most bioremediation technologies run under aerobic conditions, but running a system under anaerobic conditions (Colberg and Young, 1995; Shukla et al., 2010) may allow microbial organisms to degrade otherwise recalcitrant molecules.

2.2.3 Factors of Bioremediation

The control and optimization of bioremediation processes require a complex system of many factors. These factors include: the existence of a microbial population

capable of degrading the pollutants, the availability of contaminants to the microbial population, the environment factors (type of soil, temperature, pH, the presence of oxygen or other electron acceptors, and nutrients) (Vidali, 2001).

i. *Microbial populations for bioremediation processes*

Microorganisms could be isolated from almost any environment. Microorganisms can adapt and grow at freezing temperatures, as well as at extreme heat, in water, aerobically, and in anaerobic conditions, with the presence of toxic compounds or on any waste stream. The main requirements for microbes are an energy source and a carbon source (Vidali, 2001).

Petroleum hydrocarbons can be degraded by various microorganisms such as bacteria, fungi, yeast and microalgae (Riser-Roberts, 1992). The most important factor for petroleum biodegradation is the ability of microorganisms to utilize hydrocarbons for their cell growth and energy needs. A large number of research reported that low molecular weight alkanes are the most rapidly degraded. Mixed cultures are more effective in biodegradation of petroleum than pure cultures (Gerdes et al., 2004; Ghazali at el., 2004).

In many environments, there is already an adequate indigenous microbial population capable of extensive oil biodegradation, provided that environmental conditions are proffered for oil-degrading metabolic activity (Capelli et al., 2001; Kim et al., 2004). There are many advantages for indigenous microorganisms rather than adding microorganisms to degrade hydrocarbons. First, natural communities have developed through many years. These microorganisms are adapted for proliferation and survival in that environment. Secondly, the ability to utilize hydrocarbons is distributed among various microbial communities. This population occurs in natural environments and either independently or in combination metabolizes different hydrocarbons. Often, when the amount of microorganisms is adequate in the polluted environment, microbial seeding is not necessary. Availability of nutrient, particularly of nitrogen and phosphorus, is the most limiting factor. It was proved that these nutrients increase growth of microorganisms, which cause more rapid break down of contaminants

(Chaineau et al., 2005; Coulon et al., 2004). Microorganisms can be subdivided into the following groups:

a. Aerobic

The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions (Das and Chandran, 2011). In the presence of oxygen, examples of aerobic bacteria which are known for their derivative abilities are *Pseudomonas*, *Alcaligenes*, *Sphingomonas*, *Rhodococcus*, and *Mycobacterium*. These microorganisms are reported to degrade pesticides and hydrocarbons; alkenes and polyaromatic compounds. These bacteria use the contaminant as the sole source of carbon and energy (Vidali, 2001). Aerobic biodegradation of hydrocarbons and crude oil is well known and it is a well-studied process. The ability of anaerobic microorganisms to oxidize and use crude oil as a complex organic substrate under aerobic conditions is also discovered. These microorganisms degrade most organic compounds into carbon dioxide, water and mineral, as sulfate, nitrate and other inorganic compounds. Hydrogen sulfide or methane is not produced as reaction products. The aerobic pathway is more rapid and more efficient, because aerobic reactions consume less free energy for initiation and yield more energy per reaction (Hamme et al., 2003). Figure 2.2 shows the principle of hydrocarbons aerobic degradation. The initial organic pollutants intracellular attack is an oxidative process, the activation and oxygen incorporation is the enzymatic key reaction catalyzed by peroxidases and oxygenases (Das and Chandran, 2011).

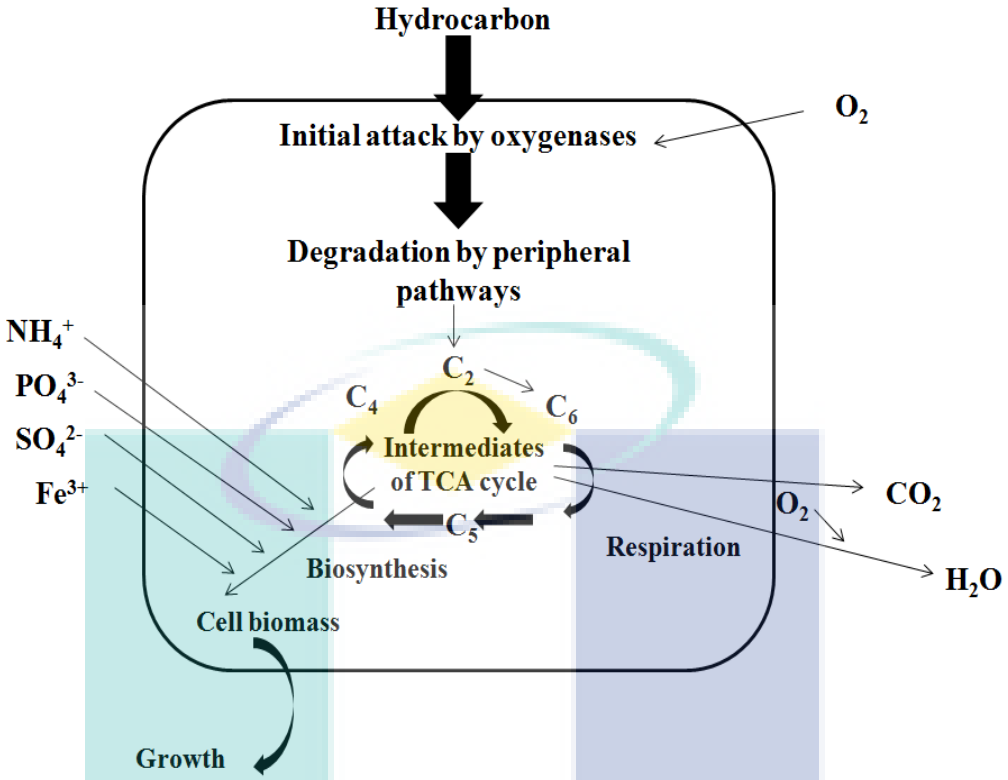


Figure 2.2 Main principle of aerobic degradation of hydrocarbons by microorganisms

Source: Das and Chandran (2011)

The hydrocarbons are degraded by a series of reactions mediated by enzymes. Oxygen works as an external electron acceptor, while an organic component of the contaminating materials serves as the electron donor or energy source. The overall degradation pathway for alkene includes sequential production of an alcohol, an aldehyde and a fatty acid. The fatty acid is cleaved, releasing carbon dioxide and forming a new fatty acid that is two carbon units shorter than the original molecule, known as beta-oxidation. The primary enzymatic attack includes a group of monooxygenases (Hamme et al., 2003).

b. Anaerobic

Anaerobic bacteria are not as frequently used as aerobic bacteria. There is an increasing interest in anaerobic bacteria used for bioremediation of polychlorinated

biphenyls (PCBs) in river sediments, dechlorination of the solvent trichloroethylene (TCE), and chloroform (Vidali, 2001). In contrast to the extensive investigation that has been done for aerobic microbial hydrocarbon metabolism, the same is not true about anaerobic hydrocarbon metabolism. The roles of bacteria that take part in these processes under anoxic/anaerobic conditions during biodegradation are not completely understood. Oxygen cannot be available in all environments where hydrocarbons occur (e.g. in deep sediments, flooded soils, entropic lagoons, stagnant fresh and ocean waters and in oil reservoirs). Many studies have investigated the problem, whether or not the biodegradation of hydrocarbons can be carried out under anoxic conditions. In late 1980s, a new group of microorganisms was found to degrade hydrocarbons under strictly anoxic conditions. Research studies have proved that these microorganisms activated organic compounds by unique biochemical mechanisms that were completely different from those employed in aerobic hydrocarbon metabolism, which were known as anaerobic biodegradation (Riser-Roberts, 1992) (Figure 2.3).

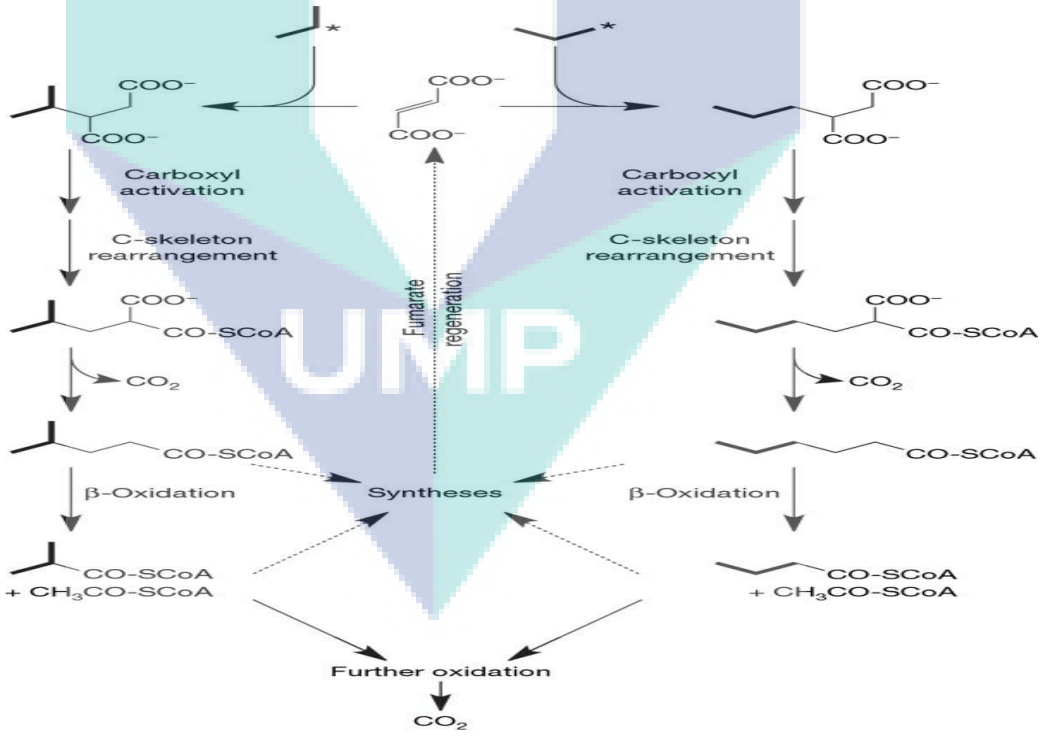


Figure 2.3 Anaerobic activation reactions of alkane

Source: Kniemeyer et al. (2007)

c. Methylotrophs

Methanotrophs are a specific subset of methylotrophs which are aerobic bacteria that can grow on methane. They have been studied for their potential to be used directly in bioremediation due to the initial enzyme in the aerobic degradation pathway, methane monooxygenase enzyme(s) they possess. These oxygenases have broad substrate specificity and have been shown to co-oxidise pollutants such as aromatics (Jechorek et al., 2003), TCE (trichloroethylene) (Bowman et al., 1993) and chlorinated aliphatic trichloroethylene and 1, 2-dichloroethane (Vidali, 2001).

d. Fungi

Some fungi such as the white rot fungus *Phanaerochaete chrysosporium* are able to degrade extremely diverse, persistent or toxic environmental contaminants (Vidali, 2001). The filamentous fungi have good potential agents of degradation, once those microbes ramify on the substratum, digesting it by the secretion of extracellular enzymes. In addition, the fungi are able to grow under stressed environmental conditions, for example: low pH values, poor in nutrients and low water activity. Many authors made lists of bacteria and fungi genera which are able to degrade a wide spectrum of contaminants, from marine environment to soil (Juhasz and Naidu, 2000; Yateem et al., 1998). Among the filamentous fungi, *Trichoderma* and *Mortierella* species are the most common fungi isolated from the soil. *Aspergillus* and *Penicillium* species were isolated frequently from marine and terrestrial environments (Bonaventura and Johnson, 1997). For degradation, it is important that bacteria and the contaminants are in contact. This is not easy, as the microbes and contaminants are not uniformly spread in the soil. Some bacteria are mobile and show a chemotactic response, sensing the contaminant and moving toward it. Other microorganisms such as fungi grow in a filamentous form to the contaminant. It is possible to promote the mobilization of the contaminant by using some surfactants such as sodium dodecyl sulphate (SDS) (Vidali, 2001). Microorganism used widely as hydrocarbon biodegraders are listed in (Table 2.1).

Table 2.1: Microbes used in bioremediation

Bacteria	Fungi
Achromobbacter	*Allesheria*
Acinetobacter	*Aspergillus*
Actinomyces	*Aureobasidium*
Aeromonas	*Botrytis*
Alcaligenes	*Candida*
Arthrobacter	*Cephaiosporium*
Bacillus	*Cladosporium*
Beneckea	*Cunninghamella*
Brevebacterium	*Debaromyces*
Coryneforms	*Fusarium*
Erwinia	*Gonytrichum*
Flavobacterium	*Hansenula*
Klebsiella	*Helminthosporium*
Lactobacillus	*Mucor*
Leucothrix	*Oidiodendrum*
Moraxella	*Paecylomyces*
Nocardia	*Phialophora*
Peptococcus	*Penicillium*
Psedomonas	*Rhodosporidium*
Sarcina	*Rhodotorula*
Spherotilus	*Saccharomyces*
Spirillum	*Saccharomycopisis*
Streptomyces	*Scopulariopsis*
Vibrio	*Sporobolomyces*
Xanthomyces	*Torulopsis*
	Trichoderma
	Trichosporon

Source: (Gordon, 1994)

2.2.4 Environmental Requirements

Microbial growth and activity are easily affected by pH, temperature, and moisture. Microbes were also isolated in extreme conditions, but most of them grow optimally over a narrow range, which make it very important to achieve optimal conditions. Temperature affects biochemical reactions rates, and the rates of many of them can be doubled at10-°C rise in temperature. Over a certain temperature, the cells die. Water is essential for all the living organisms, and humidity is needed to get the optimal moisture level (Vidali, 2001). The optimum environmental conditions for the contaminants degradation are reported in (Table 2.2).

Table 2.2: Environmental conditions affecting crude oil degradation

Parameters	Condition required for microbial activity	Optimum value for oil degradation
Soil	Moisture 25–28% of water holding capacity.	30–90%
Soil pH	5.5–8.8	6.5–8.0
Oxygen content	Aerobic, minimum air-filled pore space of 10%	10–40%
Nutrient content	N and p for microbial growth	C:N:P = 100:10:1
Temperature (°C)	15–45	20–30
Contaminants	Not too toxic	Hydrocarbon 5–10% of dry weight of soil
Heavy metals	Total content 2000 ppm	700 ppm
Type of soil	Low clay or silt content	

Source: Vidali (2001)

The amount of available oxygen will determine whether the system is aerobic or anaerobic. Hydrocarbons are readily degraded under aerobic conditions. To increase the oxygen level in the soil, it is possible to sparge air. Hydrogen peroxide or magnesium peroxide is introduced in the environment in some cases. Soil structure controls the delivery of air, water, as well as nutrients. To improve soil structure, gypsum or organic matter can be applied. Low permeability of soil can impede movement of water, nutrients, and oxygen; and soils with low permeability are not suitable for *in situ* clean-up techniques (Vidali, 2001).

i. Temperature

Biodegradation of petroleum is affected by temperature. Temperature influences the physical properties and chemical composition of the oil, hydrocarbon metabolism rate by microbial population, and microorganism composition (Venosa and Zhu, 2003). Biodegradation of hydrocarbon can occur over a certain range of temperatures. The rate of biodegradation is generally reduced with the temperature decreasing. Highest rates of degradation occur at 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 temperature effect is also complicated by other factors such as the microbial population composition (Zhu et al., 2001**).**

Season and climate have to be selected for different population of hydrocarbon-utilizing microorganisms, which are adapted to ambient temperatures (Leahy and Colwell, 1990). The drying up effect is harsh on the growth and activation of microorganisms. According to Dibble and Bartha (1979), decreasing the moisture content of the soil in the dry season diminishes rates of degradation, as a result of inadequate supply of water to sustain proliferation, metabolism or both. It was indicated in a study, that temperature of soil affected TPH rate of degradation. When the temperature was set at 30-ºC, TPH rate reached 80%, when the temperature was set at 20-ºC, the rate of TPH lowered to 60%. This suggested that higher temperature enhanced degradation of TPH and accelerated bioremediation (Huang et al., 2009).

ii. Oxygen

In general, aerobic conditions are considered necessary for extensive oil hydrocarbons degradation in the environment since main degradative pathways for both saturates and aromatics include oxygenases. Normally, conditions of oxygen limitation do not exist in the upper levels of the water column in freshwater and marine environments and in the surface layer of most beach environments. It may become less in subsurface sediments, anoxic region of water columns, and most fine-grained marine shorelines, wetlands, freshwater, mudflats, and salt marshes. Factors that may affect the oxygen availability also involve the action of wave and water flow, the oil physical state, and the amount of available substrates. New studies have indicated that anaerobic metabolism of hydrocarbon may be an important process in certain conditions (Head and Swannell, 1999).

Biodegradation of some aromatic hydrocarbons such as BTEX compounds, were clearly demonstrated to be carried out under a variety of anaerobic conditions (Leahy and Colwell, 1990; Zhu et al., 2001). The possibility of diesel fuel degradation under anaerobic conditions was studied. This was to determine the extent of degradation of diesel fuel in soil columns under various anaerobic conditions. Diesel fuels were significantly degraded under all conditions in comparison with soil column with no electron supplemented (natural attenuation). However, the diesel degradation rate under

mixed electron acceptor conditions was the highest followed by sulfate reducing, nitrate reducing, and methanogenic conditions (Boopathy, 2004).

Aerobic biodegradation causes characteristic changes in petroleum hydrocarbons composition. Linear alkanes and small aromatic hydrocarbons are degraded most easily, then branched alkanes and polycyclic aromatic hydrocarbons, followed by alkylated polycyclic aromatic hydrocarbons and saturated ring compounds (Prince et al., 2003). To determine the effect of oxygen on biodegradation, polluted soil was incubated at 27°C either as well-aerated surface soil or subsurface soil with poor aeration. Jet fuel disappeared from soil surface more rapid than it did from soil subsurface. Microbial numbers and mycelial length were increased in surface soil, by 2 to 2.5 orders of magnitude because of jet fuel pollution alone and by 3 to 4 orders of magnitude because of the combination of jet fuel pollution and bioremediation. Oxygen limitation highly reduced microbial responses to jet fuel in subsurface soil. An increase in the numbers of hydrocarbon degraders was accompanied by a decrese in other aerobic heterotrophs, so there was a little change in total plate counts. The correlations between residues of hydrocarbon, numbers of microorganisms, and microbial activity help in clarifying microbial role in jet fuel removal from soil (Song and Bartha, 1990). To optimize the biodegradation of effluent polluted with gasoline and diesel, the effects of aeration interval and agitation speed was examined. The most efficient aeration interval was 33 h and agitation speed was 110 rpm causing TPH elimination of 75.9% after only 3 days. Comparative studies carried out under constant aeration (CA), without aeration (WA) and intermittent aeration (IA) conditions. Irregular aeration resulted in the highest TPH removals with 90% reduction after 22 days. The degradation percentages were 99.6% under the IA condition for the paraffins, 95.4 % for the olefins, 94% for the isoparaffins, 83.4% for the aromatics and 70.8% for the naphthenics (Vieira et al., 2009).

iii. Nutrients

Phosphorus, nitrogen, and iron play more important role than oxygen in limiting the biodegradation rate in marine waters. Nitrogen and Phosphorous are usually present in sufficient amounts in the natural environment, because they are minor trace elements. Nutrients adjustment in a high dose can enhance initial rate of oil degradation (Oh et al.,

2001). These substances are often deficient in marine and other ecosystems because non-oil degrading microorganisms (including phytoplankton) utilize them in competition with the oil degrading species (OTA, 1991). Other researchers indicated no elevation in rates of biodegradation or an increase only after a delay of several months to a year when fertilizer were used (Leahy and Colwell, 1990).

Additions of nutrient were applied to overcome a critical rate-limiting factor in aerobic oil polluted marine ecosystem. Stimulation rates have been about 3-5 times the natural rates of oil biodegradation. Larger enhancement may be achieved by addition of higher levels of nutrient, but this may risk environmental side effects as toxicity to marine creatures and eutrophication with associated algal blooms. Due to its effectiveness, bioremediation became the main treatment technique for cleaning up oil contaminants from the polluted shorelines (Atlas and Cerniglia, 1995).

When a massive oil spill happens in freshwater and marine environments the carbon supply increases dramatically and the nitrogen and phosphorus availability generally becomes the limiting factor for degradation of oil (Leahy and Colwell, 1990; Venosa and Zhu, 2003; Zhu et al., 2001). The bioremediation strategy by fertilization within the marine ecosystem is supposed to be difficult, particularly in open waters, where the water soluble nutrients concentrations would possibly decrease rapidly by dilution (Lee and Merlin, 1999).

In a study conducted in the Republic of Korea, biodegradation was stimulated by adding a small amount of yeast extract compared with biodegradation in minimal salts basal (MSB) agar. Degradation occurred quickly in slurry systems amended with sterile soil solids but not with aqueous soil extract in the same study. However, if soil was firstly combusted to eliminate organic matter, the stimulation effect on BTEX biodegradation would get lost, showing that some components of insoluble organic compounds were nutritionally useful for degradation of BTEX (Kim et al., 2008). In a different study, it has been indicated that fertilization with NPK significantly enhanced biodegradation especially that of benzene and toluene, and biodegradation was inhibited by oleophilic fertilization (Margesin et al., 2003). It has been also reported that adding nutritive salts (NPK minor amounts of citric acid) enhanced the aerobic degradation of

13 selected polycyclic aromatic hydrocarbons using the *Salmonella yphimurium* test (Dahi and Jensen, 1997). Moreover, a study was carried out to investigate the effect of the added NPK level on biodegradation of diesel oil polluted soil;, the result indicated that the microbial activity was enhanced in general as measured by soil respiration (Jørgensen et al., 2000). The effect of nitrogen concentration on synthetic effluent was evaluated. It has been shown that of the studied parameters, the ones that had the biggest influence on the total petroleum hydrocarbons (TPH) removal were nitrogen concentration of 550 mg/L and fuel concentration of 4% (v/v) (Vieira et al., 2007). A separate study found that degradation of Phenanthrene was stimulated by the addition of yeast extract, acetate, glucose or pyruvate individually (Yuan et al., 2000). Fertilization with NPK showed effect on the enhancement of the biodegradation of BTEX compounds, especially that of benzene and toluene (Margesin et al., 2003). Different kinds of mineral nutrients (NO_3-N, NH_4-N and PO_4-P) were added to the simulated oil-contaminated seawater for stimulating biodegradation of oil in the N/P ratio 10:1 and 20:1. Although naturally occurring microorganisms are capable to degrade oil, adding nutrients accelerated rates of biodegradation significantly (Xia et al., 2005). A study tested the hydrocarbons biodegradability from petroleum tank bottom sludge on soil, and it has been concluded that the rate and the quantity of degraded hydrocarbons was increased by fertilization. As a result, 40% of hydrocarbons were degraded in 30-90 days (Ferrari et al., 1994).

iv. Other factors

Other important factors that affect petroleum hydrocarbons biodegradation involve pH and salinity. There are few published research papers which study salinity effects on the microbial degradation of hydrocarbons. The metabolism rates of hydrocarbon reduced with salinity increase in the range 3.3 to 28.4 % and the results were due to general microbial metabolic rates reduction (Leahy and Colwell, 1990). Alterations in salinity may affect biodegradation of oil via changing the microbial community. Estuaries may represent a special case because values of salinity, in addition to oxygen and nutrient levels, are very different from those in coastal or ocean areas. However, there is no evidence to indicate that microorganisms are adversely affected by other than hyper-saline environment. Several freshwater organisms can

survive for a long time in seawater despite a few which can proliferate. In contrast, the optimum salinity range for most of marine species range from 2.5 to 3.5 % and grow poorly or not at all at salinity lower than 1.5 to 2 % (OTA, 1991; Venosa and Zhu, 2003).

Seawater pH is stable and slightly alkaline in general. In contrast, freshwater and soil environments pH can be widely varied. In wetlands organic soils are often acidic, while the conditions of mineral soils are more neutral and alkaline. Neutral pH is generally preferable by most of heterotrophic bacteria and fungi, with fungi being more tolerant of acidic conditions. Research studies have indicated that oil degradation increases with increasing pH, and under slightly alkaline conditions the optimum degradation occurs (Zhu et al., 2001). The significance of pressure as a variable in the hydrocarbons biodegradation is most probably limited to the deep-sea environment (Leahy and Colwell, 1990; OTA, 1991). Many strategies are based on various methodologies application to increase the biodegradation process rate or extent (Zhu et al., 2001).

2.2.5 Bioremediation Strategies

The main bioremediation goal is to degrade organic contaminants to undetectable concentrations, or at least, to concentrations below the established limits as acceptable or safe by regulatory agencies. Bioremediation is used for the chemicals degradation in soils, groundwater, wastewater, sludge, industrial wastewater systems, and gases. Because pollutants are widespread, constituting health or ecological hazards, and susceptible to microbial detoxification, huge interest has been directed at oil and oil products, gasoline, polycyclic aromatic hydrocarbons. A variety of bioremediation strategies and procedures have been used, and a number of new and promising approaches have been suggested (Okoh and Trejo-Hernandez, 2006). Several techniques are applied depending on the saturation and aeration degree of the targeted area;, Figure 2.4 shows different types of bioremediation activities. In situ bioremediation are defined as the application of the strategy on soil and groundwater at the site with the least disturbance. Ex situ bioremediation are techniques that are applied to the removed soil and groundwater from the site via excavation (soil) or pumping (water). On the other

hand, bioaugmentation techniques include adding the microbes which have the ability to degrade contaminants (Vidali, 2001).

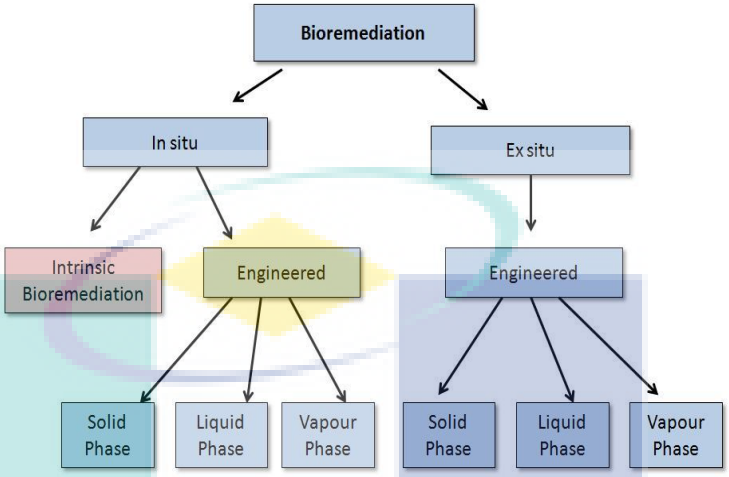


Figure 2.4 Different types of bioremediation activities

Source: Santra (2010)

A. In Situ Bioremediation

Generally, this technique is the most desirable option because of its low cost and minimum disturbance level since the application is done in the places which avoid excavation and contaminants transport. In situ technique depends on the depth of the soil that can be treated effectively. In many soils effective oxygen diffusion for preferable bioremediation rates extend to a few centimeters only to about 30 cm into the soil, despite 60 cm depths and greater were effectively treated in some cases (EPA, US, 1990). The most important in situ applications are:

i. Bioventing

It is the most common in situ bioremediation technique. It includes supply of air and nutrients via wells to polluted soil to stimulate the indigenous bacteria (Figure 2.5). This treatment employs low air flow rates providing the amount of oxygen necessary for the biodegradation only and minimizing volatilization and contaminants released to the

environment. It is efficient for simple hydrocarbons and it is useful when the contamination is deep under the surface (EPA, US, 1996).

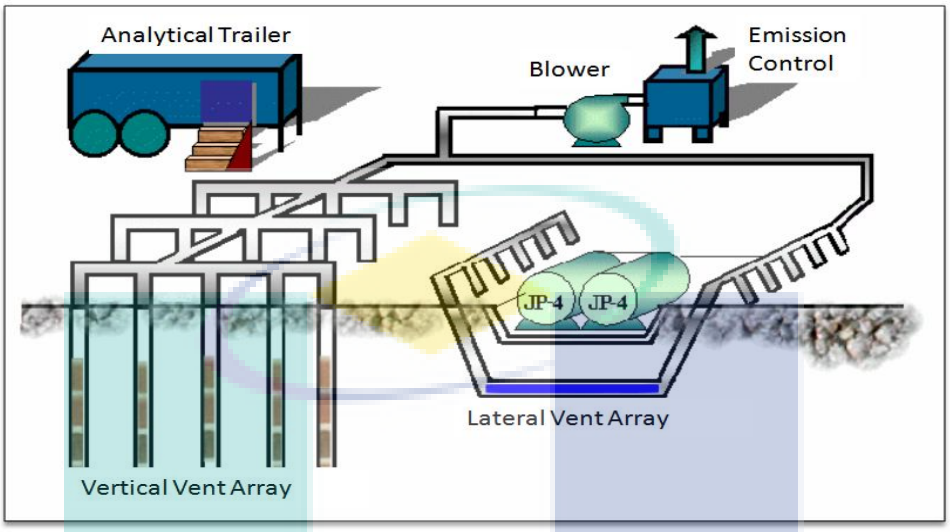


Figure 2.5 Typical Bioventing system

Source: Santra (2010)

ii. In situ biodegradation

It involves oxygen and nutrients supply by circulating aqueous solutions through polluted soils to stimulate indigenous bacteria to degrade organic pollutants. It can be used for groundwater and soil. This technique involves conditions such as the infiltration of water-containing nutrients, oxygen and electron acceptors for groundwater treatment (EPA, US, 1996).

iii. Biosparging

It involves air injection under pressure below the water table to elevate oxygen concentrations of groundwater and to enhance biological degradation rate of contaminants by naturally occurring bacteria. This technique increases the mixing in the saturated zone and consequently increases soil and groundwater contact (EPA, US

1996). The ease and low costing of installing small diameter air injection points allows considerable design flexibility and construction of the system (Figure 2.6).

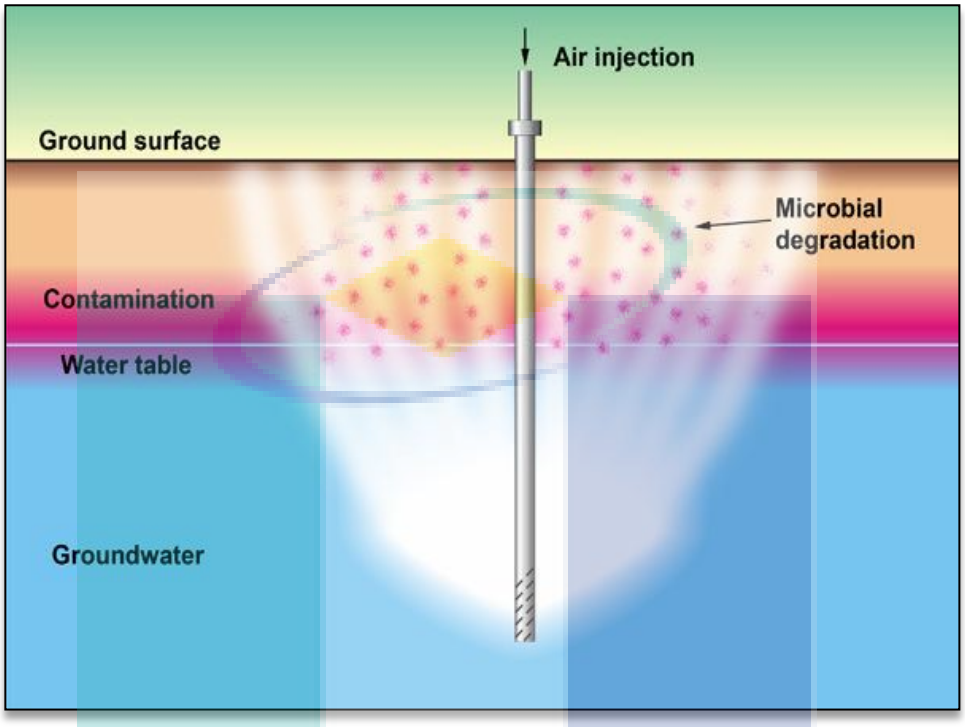


Figure 2.6 Biosparging enhances the microbial degradation

Source: Santra (2010)

iv. Bioaugmentation

Bioremediation involves adding indigenous or exogenous microorganism to the contaminated sites. The addition of microbial cultures in a land treatment unit is affected by two factors; non-indigenous cultures cannot sufficiently compete with an indigenous population to develop useful population levels and most soils with exposure to biodegradable waste for a long time have indigenous microorganisms that are effective degrades (EPA, US., 1996). Bioaugmentation could be performed under both aerobic and anaerobic conditions .Adding nutrients, electron acceptors, or electron donors can also be necessary (Figure 2.7).

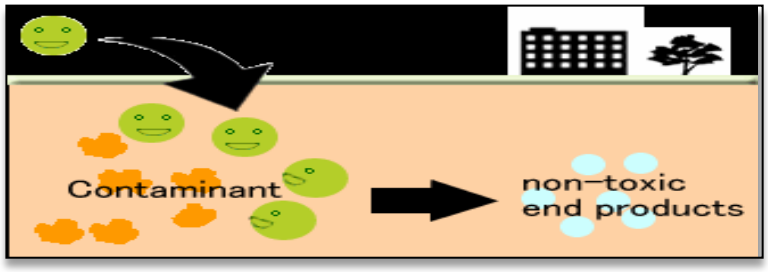


Figure 2.7 Biaugmentation

Source: Santra (2010)

v. Bioslurping

According to Santra, 2010, bioslurping is a unique technique, where it also treats free product phases floating on the groundwater top. This technique includes application of vacuum to extract soil vapor, water, and free product from the subsurface. Then, those substances are separated and disposed of properly (Figure 2.8).

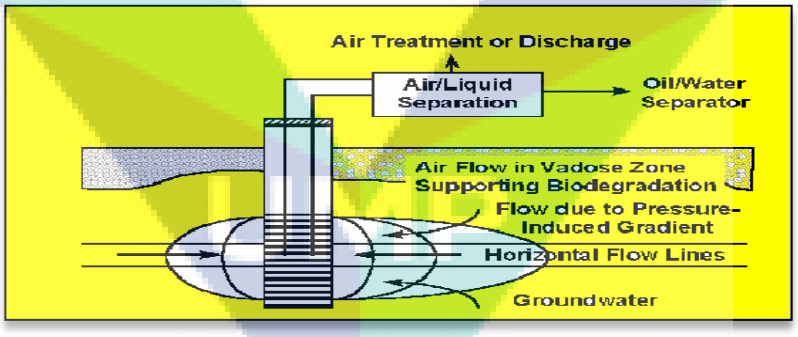


Figure 2.8 Typical in situ bioslurping system

Source: Santra (2010)

B. Ex Situ Bioremediation

These techniques are based on the removal of contaminated soil from the site (Vidali, 2001). Ex situ bioremediation is considered the optimal biodegradation

technology, because of its potential to control basic parameters and cause fast kinetics of pollutant biodegradation. Among these are the following: easy technical supervision of decontamination process; profound acceleration of pollution removal due to possibility of easy implementing a variety of necessary technical actions; easy monitoring, assessment and setup of conditions favorable for the development of microorganism cultures within the soil environment such as oxygen and nutrients. On the other hand, the costs are relatively high, due to the need to remove and transport large amount of soil physically (Kaszycki et al., 2011).

i. Landfarming

Polluted soil is excavated and spread over a bed already prepared and tilled periodically until contaminants are degraded (Figure 2.9). The aim is to stimulate indigenous biodegradative microbes and promote their aerobic degradation of pollutants. Generally, the application is limited to the treatment of superficial (10–35 cm) of soil. Because landfarming has the potential to decrease maintenance and monitoring costs, and clean-up liabilities, it has got much attention as alternative for disposal (Vidali, 2001).

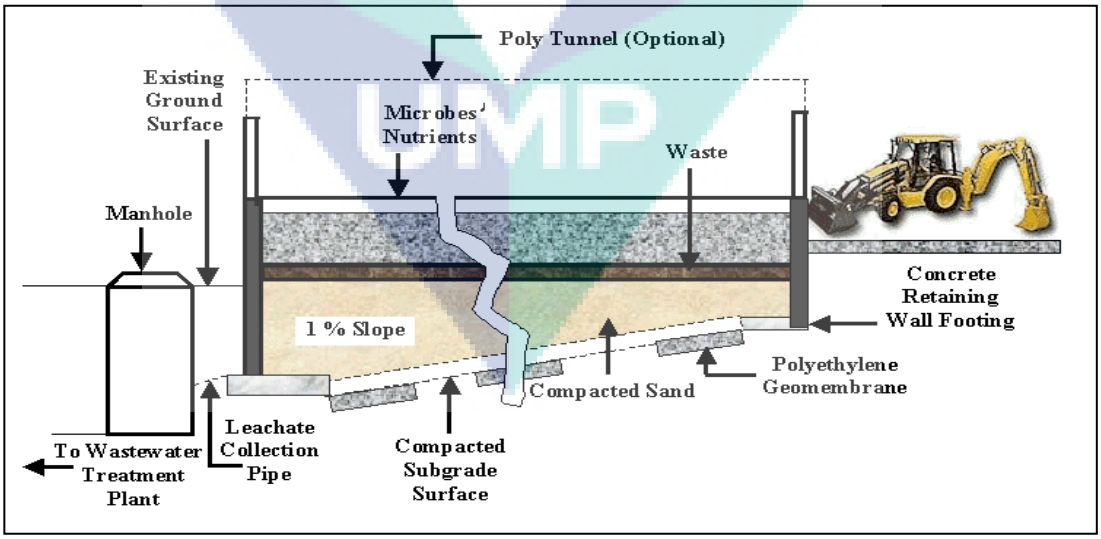


Figure 2.9 Typical land farming treatment unit

http://www.frtr.gov/matrix2/section4/D01-4-12.html

ii.Composting

This technique involves combining polluted soil with nonhazardous organic amendants such as agricultural wastes or manure. Presence of these organic substances supports rich microbial population development and increase temperature characteristic of composting (Figure 2.10) (Vidali, 2001).

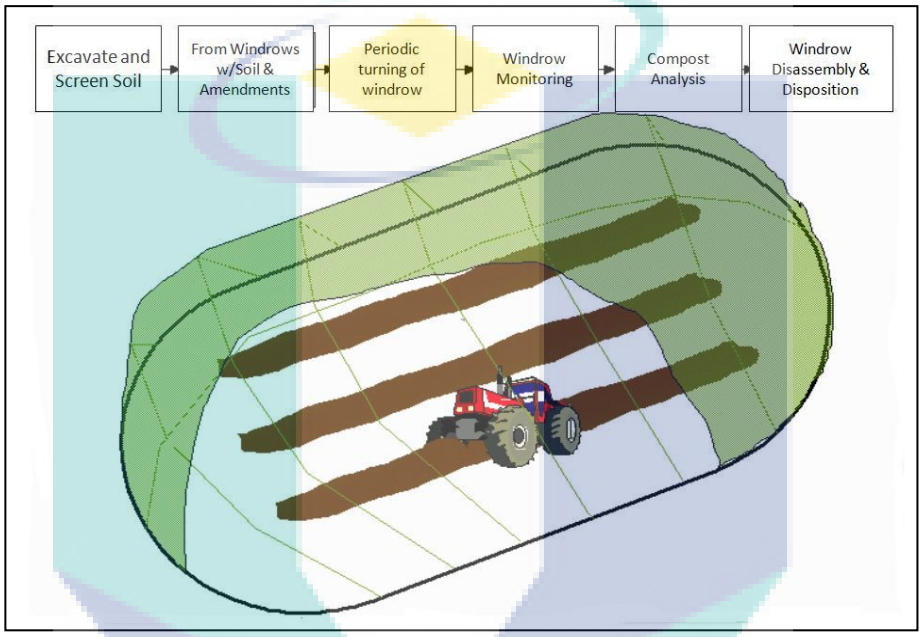


Figure 2.10 Typical windrow composting process

Source: Santra (2010)

iii. Biopiles

It is a hybrid of landfarming and composting (Figure 2.11). Engineered cells are constructed as aerated composted piles. It is used for surface contamination treatment with petroleum hydrocarbons; they are refined version of landfarming that tend to control contaminants physical losses by leaching and volatilization. Biopiles provide optimum environment for indigenous aerobic and anaerobic microorganisms (Fahnestock et al., 1998).

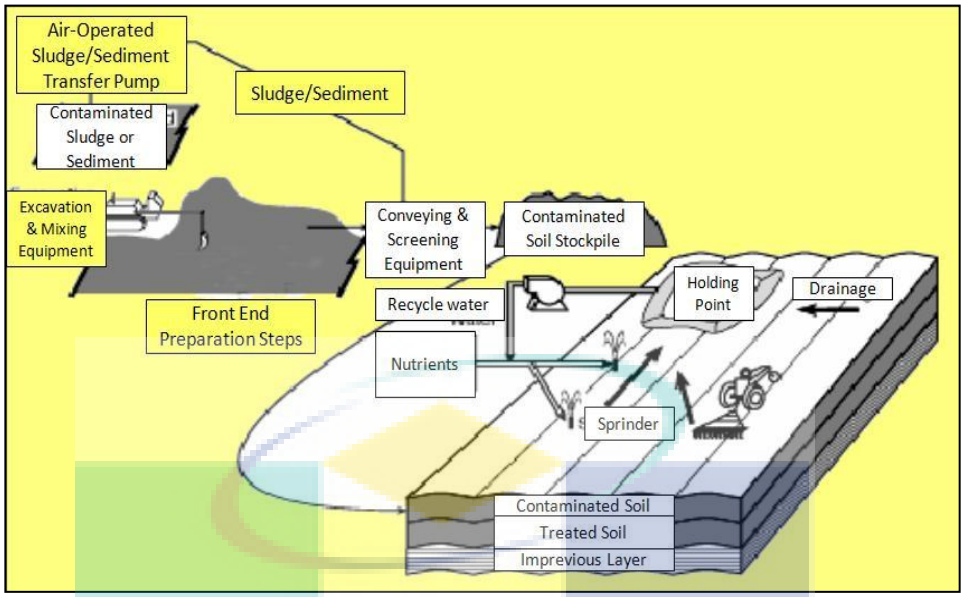


Figure 2.11 Typical biopile for solid phase bioremediation

Source: Santra (2010)

iv. Bioreactors

Slurry or aqueous reactors are used for ex situ treatment of polluted soil and water pumped up from a contaminated site (Figure 2.12). Bioremediation in reactors includes the processing of polluted solid material such as soil, sediment, and sludge or water through an engineered containment system. A slurry bioreactor can be defined as a containment apparatus used to create a three-phase (solid, liquid, and gas) mixing condition to elevate the bioremediation rate of soil bound and water-soluble contaminants as a water slurry of the polluted soil and biomass (indigenous microorganisms) is able of degrading target contaminants. Generally, the biodegradation rate and extent are higher in a bioreactor system compared with in situ or in solid-phase systems because the contained environment is manageable and more controllable. There are some drawbacks. The polluted soil needs pre-treatment (e.g., excavation) or the pollutants can be stripped from the soil by soil washing or physical extraction (vacuum extraction) prior placing in a bioreactor (Vidali, 2001).

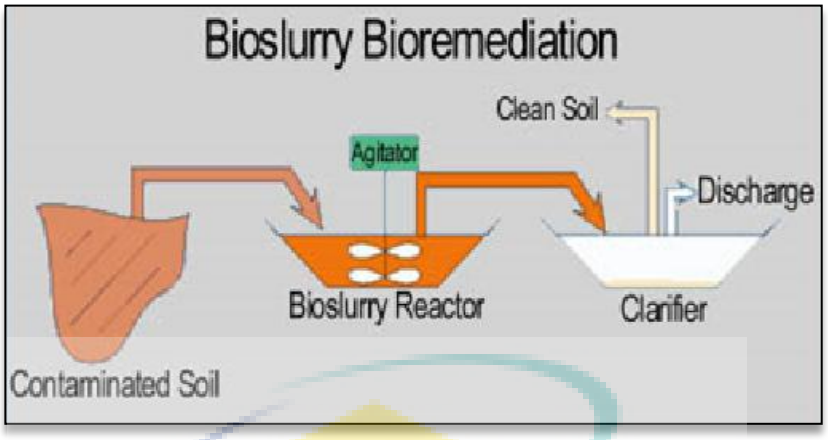


Figure 2.12 Bioslurry bioremediation system

Source: Santra (2010)

2.3 PETROLEUM HYDROCARBONS

Petroleum products are widely used as fuels, solvents and feedstocks in the textile, plastic and pharmaceutical industries. Petroleum is a complex mixture of hydrocarbons and other organic compounds, involving a number of organometallo-constituents. Petroleum compounds include: saturates, aromatics, resins and asphaltenes (Figure 2.13). Saturates are hydrocarbons with no double bonds. They are classified based on their chemical structures into: alkenes (paraffins) and cycloalkanes. Saturates represent the highest percentage of crude oil constituents. Aromatic hydrocarbons with one or several aromatic rings can usually be substituted with various alkyl groups. In comparison to the saturated and aromatic fractions, the resin and asphaltenes contain non-hydrocarbon polar compounds. Resins and asphaltenes have quite complex and generally unknown carbon structure with the addition of many nitrogen, sulfur and oxygen atoms (Harayama et al., 2004). Petroleum obtained from different sources varies widely in their composition and physical properties. The composition of certain petroleum product ranges has molecular weight ranging from 75 to 1500 (Riazi et al., 1999). The chemical structure of hydrocarbons affects its biodegradation in two different ways. First, the molecule may contain groups or substituent which cannot react with available or inducible enzymes. Second, the structure determines the physical state of the compound state where microbial degradation does not occur easily. Usually, the

oxidation of hydrocarbon will be slower as the compound is larger and more complex. The degree of substitution also affects the degradation. Compounds that contain amine, methoxy and sulfonate groups, ether linkages, halogens and branched carbon chains are generally persistent. Adding aliphatic side-chains increases the susceptibility of cyclic hydrocarbons to microbial attack (Riser-Roberts, 1992).

Physicochemical properties of hydrocarbon are affected by their composition, and solubility of hydrocarbons differ from one to another, ranging between polar compounds, such as methanol to very low solubility non-polar compounds, such as high molecular weight polynuclear aromatic hydrocarbons (PAH). The solubilization is not the only factor affecting the degradation of hydrocarbons. Several microorganisms, such as *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Bacillus laterospor* secrete emulsifiers that increase the substrate surface area. Otherwise, these microorganisms change their cell surface to increase its affinity for hydrophobic substrates and, facilitate their absorption (Carvalho and Fonseca, 2005; Cybulski et al., 2003).

Hydrocarbons can be very fluid or very viscous and very volatile or relative nonvolatile. Viscosity of contaminating oils is an important property. It determines the spreading and dispersion of the mixture of hydrocarbon as well as the surface area that can be available for microbial attack. The low concentration of organic compounds in the environment makes all fractions more susceptible to be attacked. However, at high concentrations, only those fractions most susceptible to degradation will be broken down (Doong and Wu, 1995).

Also the concentration of pollutants can affect the number of organisms present in the environment. It has been proven that the higher concentrations of gasoline in contaminated water related directly to higher counts of microorganisms (Doong and Wu, 1995). This finding was supported by a study, which result showed that the number of oil degrading microorganisms contaminated beaches in comparison with untreated controls increased by as much as 10,000 times. This was due to the availability of nutrients and the ability of microbes to degrade the contaminant, which enhanced the microbial community development (Pritchard et al., 1992).

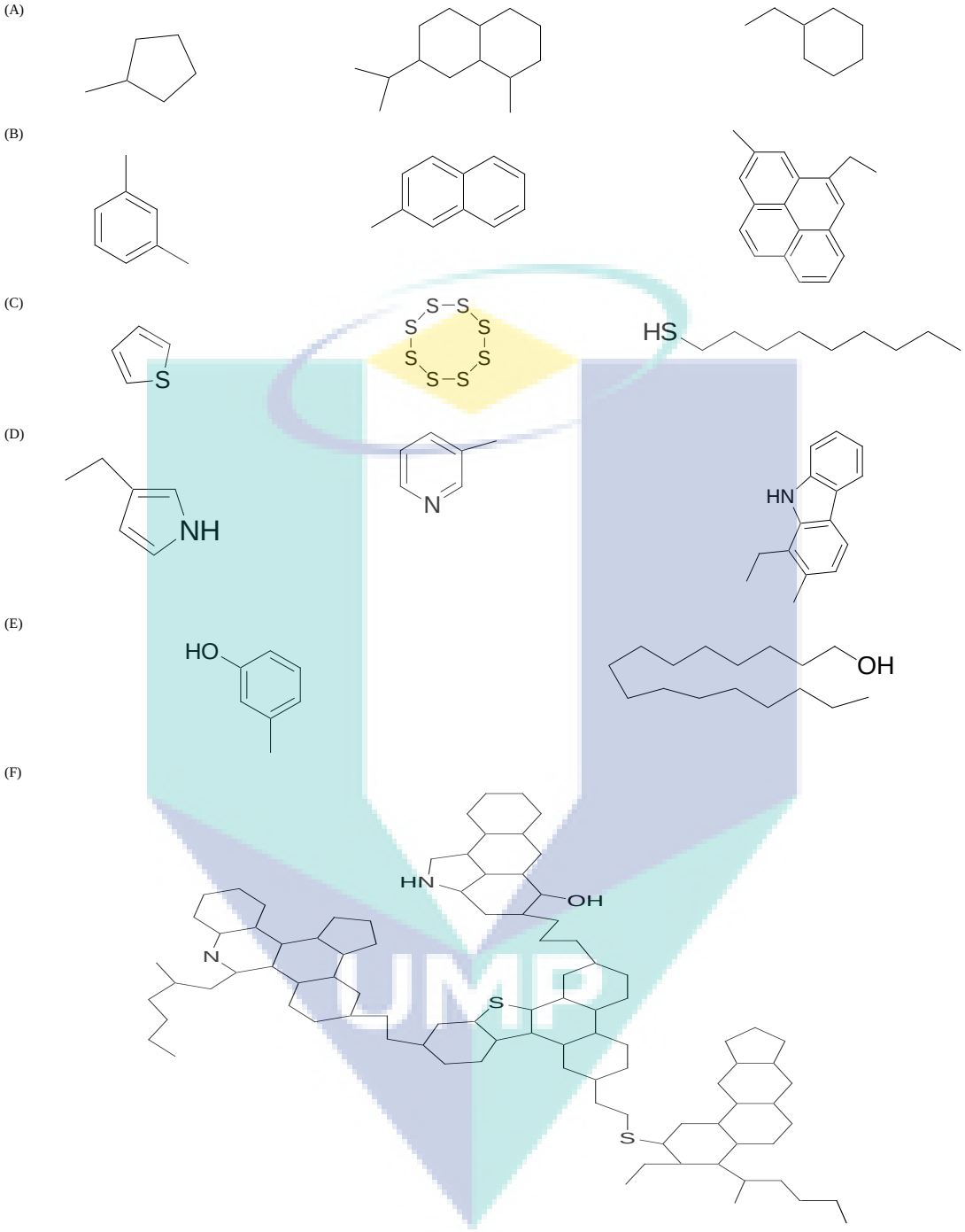


Figure 2.13 Structures of crude oil constituents: (A) substituted cyclopentane, cyclohexane, bicyclic species, (B) substituted aromatics (xylene, naphthalene, and perylene), (C) thiophene, elemental sulfur, nonyl mercaptan, (D) substituted pyridine, pyrrole, carbazole, (E) phenol, long chain alcohol, (F) asphaltene model molecule

Source: Machin et al., (2005)

2.3.1 Petroleum Hydrocarbon Hazards

Generally, spilled oil causes harmful effects to the environment when shallow, porous sediments or special-use habitats are affected. Oiling of feathers is the primary cause of bird deaths after oil spills (Irwin et al., 1997; Solomon and Janssen, 2010). Crude oil components (some PAHs like benz (a) anthracene, benzo (a) pyrene, benzo (b) fluoranthene, benzo (k) fluoranthene, chrysene, dibenz (a,h) anthracene, and indeno (1,2,3-cd) pyrene) are found to be carcinogenic in animals and it is a probable human carcinogens. Reported effects of petroleum and PAHs on living organisms include impaired immune systems for animals and changed endocrine functions for fish and birds, and it also includes impaired reproduction and decreased growth and development for plants, invertebrates, fish, reptiles, amphibians, and birds (Irwin et al., 1997). Chemicals of crude oil cause respiratory and nervous system problems. Oil can cause skin allergy and other diseases. Oil spills can also cause damage to DNA leading to mutations (Harmon, 2010).

2.3.2 Engine Oil

Engine oil is a mixture of complex hydrocarbons and other organic compounds, involving some organometallic constituents (Butler and Mason, 1997; Jain et al., 2010). It is used to lubricate automobile engines (Hagwell et al., 1992). Large amounts of lubricating engine oils are long-chain saturated hydrocarbons (base oil) and some additives. The main constituents of the base oil are cyclic alkanes. Long-chain hydrocarbons and cyclic alkanes are known as recalcitrant to microbial degradation. The base oil contains C_{16}-C_{36} hydrocarbons, and more than 75% are cyclic alkanes. The number of rings of cyclic alkanes in the base oil range from 1 to 3 and each ring contains between 5 to 6 members, mostly with long alkyl side chains. Hydrocarbon susceptibility to microbial degradation differs according to the type and size of the hydrocarbon molecule (Table 2.3). Alkanes of intermediate chain length (C_{10}–C_{24}) usually degraded rapidly, while very long chain alkanes are resistant to microbial degradation (Koma et al., 2003; Tang et al., 2007).

Used motor oil spill is an environmental hazard. Most mechanical methods aiming to reduce hydrocarbon pollution high in cost time consuming and mainly depend on soil excavating. These treatments involve incineration or burial in secure landfills (Udeani et al., 2009). These are effective methods but after burning, soil will lose its structure and nutritional value. These treatments do not remove the pollution but only decrease the problem (Lageman et al., 2005). Bioremediation techniques were effective methods that stimulate the biodegradation of polluted soil (Mishra et al., 2001) and could restore polluted soils by the broad biodegradative abilities of microorganisms towards organic compounds (Thenmozhi et al., 2011).

Table 2.3: Chemical composition of car engine base oil

Component	**(%)**
Saturated fraction	90.9
Normal paraffin	15.5
Cyclic paraffin	75.4
Aromatic fraction	9.1
Naphthalene	1.7
Fluorene	1.2
Benzene	1.1
Dibenzofuran	1.0
Dinaphthenebenzene	0.8
Dibenzanthracene	0.6
Naphthobenzothiophene	0.3
Perylene	0.2
Benzothiophene	0.2
Chrysene	0.1
Unknown	1.9

Source: Koma et al. (2001)

2.3.3 Dangers for Soil, Water, Air and Health

When used motor oil is disposed improperly, there will be a risk that it could enter natural cycles and the food chain through water, soil and air. Hydrocarbons, heavy metals, and other halogen compounds contained in used oil pose risks on human health and can impede growth of plant and their ability to absorb water. One liter of used oil can contaminate one million liters of groundwater or surface water. This is because the concentration of oil in the water is then 1 ppm, a level that is seen as the upper limit that can still be considered tolerable (Chung et al., 2007; Porst, 2000).

Used engine oil contains metals and heavy polycyclic aromatic hydrocarbons (PAHs) which contribute to chronic hazards such as mutagenicity and carcinogenicity (Keith and Telliard, 1979). Chronic exposure to high concentration of oil may develop liver or kidney disease and an increased risk of cancer (Propst et al., 1999).

2.3.4 Used Motor Oil Contents

Used engine oil contains much higher concentrations of PAHs compared with new motor oil (Irwin et al., 1997). The concentration of PAHs in used oil may range from 34 to 190 times higher than those in fresh motor oil (Grimmer et al., 1982). Used engine oils contain greater percentage of aromatic and aliphatic hydrocarbons (C_{15}-C_{50}), nitrogen and sulphur compounds, in addition to metals such as (Mg, Ca, Zn, and Pb) than fresh oils. These contaminants arise from normal wear of engine components and from heating and oxidation of lubricating oil during engine operation (Hewstone, 1994). Napththalene, acenaphthalenes, dinaphthenoanthracenes benzo[*a*]pyrene, and benzo[*a*]anthracene were detected in used oil samples by Cotton et al. (1977).

2.4 ISOLATION AND IDENTIFICATION OF MICROORGANISMS FROM CONTAMINATED SOIL

Microbial population can be found and isolated from almost any environmental conditions. Microorganisms will adapt and grow at freezing temperatures, and extreme heat, desert conditions, in water, with oxygen, and in anaerobic conditions, in the presence of toxic materials or on any waste stream. The essential requirements are a

carbon source and an energy source. Because of the adaptability of the microbes, they can be used to degrade or remediate environmental hazards (Vidali, 2001).

2.4.1 Isolation of Microorganisms from Petroleum Contaminated Soil

In several studies, bacteria were successfully isolated from soil contaminated with hydrocarbon by controlling type of media, hydrocarbon source, temperature, and period of incubation. Identification was performed using different techniques, as biochemical tests, and PCR technique. Three bacterial isolates capable of utilizing used engine-oil as a source of carbon were isolated from contaminated soils using the enrichment technique. Three isolates were identified as *Flavobacterium* spp., *Acinetobacterium calcoaceticum* and *Pseudomonas aeruginosa* according to biochemical tests and 16S rRNA sequencing (Thenmozhi et al., 2011). In another study, hydrocarbon degrading microorganisms isolated from three sites in Western New York State by selective enrichment technique, resulted in the collection of 20 different species (Malatova, 2005). However fifteen crude oil degrading *bacillus* spp. were isolated from contaminated sites. Two isolates showed best growth in liquid media with 1-3% (v/v) crude oil and mineral salt medium in a study conducted by (Sepahi et al., 2008). Furthermore three species isolated from a crude oil contaminated soil in Eket, Akwa Ibom State of Nigeria were *Micrococcus varians*, *Bacillus subtilis* and *Pseudomonas aeruginosa* (Ekpo and Udofia, 2008). In addition to that, ten indigenous microorganisms were isolated from contaminated soils using the enrichment technique. Five isolates with the highest degradation potentials under standard degradation conditions were identified as *Acinetobacter calcoaceticus (*LT1 and ETS2), *Acinetobacter* spp. (LT1A), *Citrobacter freundii* (MRC3) and *Bacillus pumilus* (JLB) (Singh and Lin 2008). As was reported by Chaerun et al. (2004), five years after the 1997 *Nakhodka* oil spill in the Sea of Japan, seven bacterial strains capable of utilizing the heavy oil spilled from the *Nakhodka* Russian oil tanker were isolated from three coastal areas (namely Katano Seashore of Fukui Prefecture, Osawa and Atake seashores of Ishikawa Prefecture) and the *Nakhodka* Russian oil tanker after a 5-year bioremediation process. Scanning and transmission electron microscopic observations showed that a high number of hydrocarbon-degrading bacteria still existed in the sites consisting of different morphological forms of bacteria, such as coccus (*Streptococcus* and *Staphylococcus*)

and bacillus (*Streptobacillus*). While, *Bacillus subtilis* DM-04 and *Pseudomonas aeruginosa* M and NM strains were isolated from a soil sample contaminated with petroleum from North-East India (Das et al., 2007). In a separate research study, bacterial cultures isolated from oil contaminated soil samples, *Micrococcus* sp. GS2-22, *Corynebacterium* sp. GS5-66, *Flavobacterium* sp. DS5-73, *Bacillus* sp. DS6-86 and *Pseudomonas* sp. DS10-129 were selected for the study based on the efficiency of crude oil utilization (Rahman et al., 2002). According to Richard and Vogel (1999), a bacterial consortium consisting of seven members was obtained by a soil enrichment procedure using diesel fuel as carbon source. Six of the seven isolates were identified according to their morphological and biochemical characteristics. Only three isolates were able to degrade the petroleum hydrocarbons tested. Other study suggested that three diesel-degrading bacterial strains, *Alcaligenes piechaudii* CC-ESB2, *Gordonia hirsuta* CC-GH1 and *Rhodococcus ruber* CC-RR1, isolated from a site contaminated with oil were tested for their hydrocarbon degrading potential (Lin et al., 2009). Furthermore, three bacterial strains (strains Q10, Q14 and Q18) were isolated and identified as *Pseudomonas* spp., *Flavobacterium* spp. and *Rhodococcus* spp., respectively and found to be hydrocarbon degraders (Lu et al., 2006). Investigations have been carried out on five microorganisms, three bacteria and two yeasts, capable of degrading Tapis light crude oil, which were isolated from soil contaminated with oil in Bangkok, Thailand. Soil enrichment culture was done by inoculating the soil in mineral salt medium with 0.5% v/v Tapis crude oil as the sole carbon source (Palittapongarnpim et al., 1998). In aprevious study, *Ralstonia picketti* SRS (BP-20), *Alcaligenes piechaudii* SRS (CZOR L-1B), *Bacillus subtilis* (I'-1a), *Bacillus* spp. (T-1), and *Bacillus* spp. (T'-1), were isolated from petroleum-contaminated soils (Plaza et al., 2008).

Single cultures of fungi were found to be better than mixed cultures of bacteria and fungi. Fungi were found to be better degraders of petroleum than bacteria (Okerentugba and Ezeronye, 2003). The filamentous fungi are good potential agents of degradation, once they ramify on the substratum, digesting it via the secretion of extracellular enzymes. In addition, the fungi are capable of growing under stress environmental conditions, such as environment with low pH values or low nutrients and with low water activity. Among the filamentous fungi *Trichoderma* and *Mortierella* spp. are the common ones isolated from the soil (Yateem et al., 1998).

Although, hydrocarbon degraders could be isolated from a petroleum oil contaminated environment, it can also be isolated from a totally unrelated environment (Ojo, 2005). In most previous studies, the source of fungi was selected from crude oil contaminated soils or water, phyloplanes, rhizoplanes, food or crops as well as root tubers. Some other sources of fungi for biodegradation of petroleum oil, are the use of mycorrhizal fungi experimentally and wood rotting fungi in petroleum biodegradation. Fungi were isolated from oil seeds in the tropical forest as petroleum oil biodegraders. It was shown that the utilization of the hydrocarbons which make up the oils by the pathogenic fungi with the assistance of the lipase enzyme they produce is the main mechanism (Adekunle and Uma, 1996). These fungi have been shown to be capable of utilizing the hydrocarbons present in petroleum (Adekunle and Oluyode, 2002).

Some fungi species such as *Trichoderma* (Cserjesi and Johnson, 1972) and *Phanerochaete* (Andrea et al., 2001) were found to be implicated in hydrocarbon biodegradation. Fungal bioremediation were successful for the clean-up of polycyclic aromatic hydrocarbon (Andrea et al., 2001). The use of fungi is relatively economical as they can grow on a number of low costing agricultural or forest wastes such as corncobs and sawdust (Bijofp, 2003).

In a study conducted by George-Okafor et al. (2009), twelve fungal isolates were recovered from oil polluted soils, and tested for crude oil biodegradation activity. Among them, only eight were found to have potentials for biodegradation and two of the eight isolated were identified as *Aspergillus versicolor* and *Aspergillus Niger*. They exhibited the fastest and the highest extent of biodegradation.

White rot fungus *Polyporus* spp. S133 was collected from petroleum contaminated soil and it was tested for its ability to grow and degrade crude oil. When *Polyporus* spp. S133 was incubated in 1000 ppm of petroleum oil for 60 days, the degradation reached 93% (Hadibarata and Tachibana, 2009). The filamentous fungi (*Aspergillus niveus*, *Aspergillus niger*, *Aspergillus versicolor*, *Aspergillus terreus*, *Aspergillus fumigatus*, *Penicillium corylophilum*, *Parcilomyces variotti*, *Paecilomyces niveus* and *Fusarium* spp. *A. versicolor*) have biodegradation ability in addition to the

organic matter removal, which could be a potential fungus to degrade petroleum hydrocarbons (Lemos et al., 2002).

2.4.2 Isolation of Microorganisms from Used Engine Oil Contaminated Soil

Many species of bacteria were isolated from used car engine lubricating oil, and then they were examined using both used and unused oils as substrate. Used oil worked as a better substrate for growth of the bacteria than fresh oil. The bacterial isolates were identified as *Bacillus, Corynebacterium, Actinomyces, Micrococcus, Serratia, Citrobacter, Edwardsiella, Pseudomonas, Nocardia* and *Acinetobacter* species (Okpokwasili and Okorie, 1988). Three bacterial spp. capable of utilizing used motor oil as a carbon source were isolated from polluted soils by the enrichment technique and were identified as *Pseudomonas aeruginosa, Serratia marcescens* and *Bacillus licheniformis* using biochemical tests and 16S rRNA sequencing. *Pseudomonas aeruginosa* and a consortium of the isolates were capable of using 81 % and 90 % of used motor oil, respectively under laboratory conditions in a 4 week period (Thenmozhi et al., 2011).

Based on screening, mixed bacteria found to be more effective than of a single strain in used car oil biodegradation. A mixed bacterial flora was isolated from the soil of two petroleum contaminated sites, cultivated in an open environment and then used to degrade used car oil in wastewater. Engine oil concentration changed the growth rate of the bacterial consortium, and the mixed bacterial consortium was found to have high biodegrading efficiency for engine oil (Liang et al., 2007). Mixed cultures and single bacterial strains, *Pseudomonas* spp., *Arthrobacter* spp. and *Mycobacterium* spp. were isolated from hydrocarbon polluted soils by enrichment on crude oil or individual hydrocarbons, as the carbon sources. The strains were selected depending on their ability to grow in media containing crude oil, used engine oil or mixture of them. Their capability to degrade hydrocarbon pollution in the environment was screened using soil samples polluted with used engine oil. The mixed culture degraded 66 % of aliphatic compounds in the motor oil, after 60 days of incubation. The other mixed starter culture removed 47 % of aromatic compounds through 60 days of incubation, which is the maximum effect among the cultures tested (Bagherzadeh-Namazi et al., 2008).

Indigenous fungal isolates were isolated from used motor oil samples. The pure fungal isolates obtained were identified as *Trichoderma asperellum, Trichoderma asperellum, Trichoderma asperellum*, *Penicillium* species, and *Aspergillus* species. *Penicillium* strain exhibited a significant degree of degradation by degrading all of the *n*-alkanes (*n*-C_{-15} to *n*-C_{-23} range) present in the used engine oil, thus of greater potential in degrading the aliphatic hydrocarbon compounds of used car oil (Husaini et al., 2008).

2.5 DETERMINATION OF THE RATE OF HYDROCARBON BIODEGRADATION

The consideration of the applicability of oil spills bioremediation is not new, and petroleum hydrocarbons biodegradation is affected by the existing microbial population in the environment. The composition of the microbial population is affected by the composition of the hydrocarbons and the environmental conditions (Bento et al., 2005).

Petroleum hydrocarbons are divided into four classes: the saturates, the aromatics, the asphaltenes (phenols, fatty acids, ketoses, esters, and porphyrins), and the resins (pyridines, quinolines, carbazoles, sulfoxides, and amides). Rates of biodegradation have been shown to be highest for the saturates, second for the light aromatics, followed by high-molecular-weight aromatics and polar compounds exerting very low degradation rates. This is not a global pattern; however, as Cooney et al. (1985) reported higher degradation of naphthalene losses than of hexadecane in water-sediment mixtures from a freshwater lake. In addition, Jones et al. (1983) noted extensive biodegradation of alkyl-aromatics in marine sediments before any detectable changes in the n-alkane profile of the crude oil was tested. Fedorak and Westlake (1981) also reported a more rapid attack on aromatic hydrocarbons during the crude oil degradation by marine microbial populations from a pristine site and a commercial harbor.

The main groups in crude oil, n-alkanes, are easily biodegraded aerobically by many pathways. A literature review on the representative hydrocarbons biodegradability in petroleum (PHCs) shows that microorganisms (bacteria and fungi) isolated from soil, sediments, and biosolids can easily metabolize compounds of chain lengths up to C_{30}-C_{44} involving n-alkanes, branched alkanes with few alkyl groups, and 1-3 ring alkylated

or nonalkylated aromatics. Generally, highly branched alkanes, cycloalkanes, 4-6 ring condensed aromatics, and alkylated thiophenes and dibenzothiophenes are metabolized partially or are recalcitrant completely. The microbial degradation extent of crude oils, fuels, and oily wastes in soils depends on the distribution of the structures of PHC, concentration, presence of a non-aqueous phase liquid (NAPL), degree of evaporative loss (weathering), and sequestration (non-bioavailability). The decrease in bulk PHC in laboratory and field trials with crude oil or refined oil products in soil are because of volatilization and biodegradation (Salanitro, 2001).

A study on the Nakhodka oil spill in the Sea of Japan in 1997, which was conducted five years after the accident, seven bacterial strains capable of utilizing the heavy oil spilled from the Nakhodka Russian oil tanker were isolated from three coastal areas. All bacterial strains isolated could utilize long-chain-length alkanes efficiently, but not aromatic, and all of them were able to grow well on heavy oil (Chaerun et al., 2004). The effect of natural attenuation, biostimulation and bioaugmentation on total petroleum hydrocarbons degradation (TPH) was evaluated. This was in soils polluted with diesel oil. Bioaugmentation indicated that the greatest degradation was in the light (C_{12} - C_{23}) fractions (72.7 %) and heavy (C_{23} - C_{40}) fractions of TPH (75.2 %) and natural attenuation was more effective than biostimulation (Bento et al., 2005). In a previous research, it was reported that petroleum oil biodegradation by a *pseudomonas* isolated from a petroleum polluted soil was instable. The isolates utilize octadecane and dodecane much better than octane, and it did not utilize hexane, benzene, kerosene, pentane, heptane or thiophenol (Emtiazi et al., 2005).

It was suggested by Setti et al. (1993) that the alkane's degradation within heavy oil by a *pseudomonas* can be divided into three categories: liquid alkenes (C_{12} to C_{16}) are degraded rapidly, followed by C_{17} to C_{28} alkenes and high-molecular-weight alkenes above C_{28}. However, the large increase in the loss rate of the higher-molecular weight alkenes (C_{26}) is interesting. It could be that another mechanism in addition to direct biodegradation, as enhanced physicochemical removal resulted indirectly by nutrient addition, is involved. It was reported by Bragg et al. (1992) that more oil was lost from a fertilized Alaskan beach than it was expected by enhanced biodegradation alone, and they assumed that responsible factor was enhanced by physical oil loss. High

concentrations of oil could be very difficult to be removed by bioremediation, although there was no quantification for the upper limit. Oil in a water-in-oil emulsion is more difficult to be treated than weathered non-emulsified oil. However, successful experiments have been carried out with oil emulsions.

In a study conducted by Rontani et al. (1985), it was noticed that degradation of asphaltenic compounds in mixed bacterial cultures was dependent on the presence of n-alkanes C_{12}-C_{18} carbon atoms in length. The trends in the rates of removal for n-alkanes observed that the disappearance of the low molecular-weight alkanes was stimulated by the addition of fertilizer. It was mostly thought that the *n*-alkanes of shorter chain length were used more easily as an energy source than were longer-chain alkanes (Riser and Roberts, 1992).

The knowledge regarding bacterial biodegradation of high molecular weight (HMW) polycyclic aromatic hydrocarbons (PAHs) was developed in the last years. Some of HMW PAH-degrading strains have been isolated and identified. In the same period, the number of HMW PAHs compounds which is known to be degraded by these isolates and consortia has also elevated, and pathways for the degradation of HMW PAHs by number of the strains have been clarified. More explorations are still needed in many areas of HMW PAHs biodegradation research. Investigations into the HMW PAHs biodegradation regulatory mechanisms, PAHs biodegradation combined with other hydrocarbons in mixtures, and the microbial interactions within PAH-degrading consortia are examples of areas where research is required (Robert and Shigeaki, 2000). There was success variable for seeding in stimulating the degradation of organic pollutants in nature. This was due to, the fact that the contaminant concentration may be very low to support the inoculum growth, the contaminant concentration can be toxic to the inoculum, the seeding by microorganisms may be susceptible to toxins that is naturally occurring in the environment, and/or the inoculum may be unable to move to the pollutant in the environment. All of these barriers could be experienced when seeding is used to remove oil spills, except that adequate oil is normally present to support the added microorganisms (Goldstein et al., 1985).

Carbon dioxide evolution experiments were used as the main evidence of microbial degradation of oil in biometric flasks. CO_2 evolution rates measurements have shown to effectively evaluate the rates of biodegradation by providing significant data in a short period of time. The residual oil chemical composition was determined by gas-chromatographic techniques. The results showed that Leepershank crude oil had the highest accumulation of carbon dioxide and the highest degradation efficiency of medium chain alkanes, whereas Mexican crude oil degradation with a composition of higher saturated and substituted hydrocarbons took longer time. Furthermore, Smakover oil biodegradation was reduced significantly as a result of high content of aromatic and cyclic hydrocarbons (Malatova, 2005).

The gravimetric analysis showed that 80 and 90 % of used engine oil were used respectively by *A. calcoaceticum* and a consortium of the isolates, under laboratory conditions at 30 °C and 160 rpm with Bushnell-Haas media in a four week period. An increase in oil degradation is correlated to the increase of cell number indicating that oil degradation occurred because of bacterial isolates. All isolates were capable of degrading the n-paraffin up to 80 % in a two week period. The optimal temperatures at which biodegradation occurred at 30-37 °C. The favor of nitrogen sources and minimal salts varied for different bacterial isolates. The results obtained explained the potential for oil bioremediation of these isolates *in situ* and/or *ex situ* (Mandri and Lin, 2007).

In a report for used car oil biodegradation by mushroom compost (Abioye et al., 2010), hydrocarbon content of the soil samples was determined by toluene cold extraction method of Adesodun and Mbagwu (2008). Soil sample was weighed into and mixed with toluene, after shaking the liquid phase of the extract was measured at 420 nm using spectrophotometer. The total petroleum hydrocarbon (TPH) in soil was estimated with reference to standard curve derived from fresh used engine oil diluted with toluene.

CHAPTER 3

MATERIALS AND METHODS

3.1 GENERAL INTRODUCTION

3.1.1 Apparatus

UV-Visible spectrophotometer 1601 (Shimadzu), shaker (Vortex, Stuart Scientific UK), magnetic stirrer, beaker (50 and 100 mL), adjustable pipettes (1-10, 10-100, and 100-1000 μL), sensitive balance (Sartorious BL 210 S) and sterile tips and pH meter (Mettler Toledo MP 220) were obtained from Faculty of Pharmacy, Universiti Kebangsaan Malaysia (UKM). Organic solvent toluene was obtained from Merck, Germany.

Colony counter (Rocker galaxy 230), fume hood (Alba Isocide), cell culture cluster - flat bottom with lid in polystyrene, autocalve and incubator (Sastec) were obtained from Universiti Putra Malaysia (UPM).

3.1.2 Soil Sample Collection

Samples of soil, contaminated with different kinds of hydrocarbons (benzene, used car oil and diesel), were collected from five car workshops (Kuala Lumpur, Selangor, Kajang, Rawang and Serdang), through simple soil sampling method (ASTM, 1998). Samples were collected randomly from superficial (0-15 cm) area of the soil where the aerobic microorganisms live more. Soil samples were labeled and kept in special sterilized containers at 4 ºC until it was transported to the laboratory.

3.1.3 Media

i. Solid media

Selective media was prepared by adding 15.0 g of agar, 0.2 g of disodium hydrogen phosphate (Na_2HPO_4) and 0.5 g of ammonium sulfate ($(NH_4)_2SO_4$) to 1000 mL of demineralized water in a container. The medium was sterilized for 20 minutes at 120 °C, cooled to 55 °C and then 10 mL of hydrocarbon sources (used car oil, crude oil and benzene) were added, individually with continual stirring.

Mineral salt media were prepared by adding 10.0 g sodium chloride (NaCl), 0.42 g of magnesium sulfate ($MgSO_4$), 0.92 g potassium chloride (KCl), 0.53 g potassium dihydrogen phosphate (KH_2PO_4), 0.42 g of ammonium nitrate (NH_4NO_3) and 15.0 g of agar to 1000 mL of demineralized water in a container. The medium was sterilized for 20 minutes at 120 °C. Nutrient agar was prepared by adding 23.0 g of nutrient agar to 1000 mL of demineralized water in a container and sterilized for 20 minutes at 120 °C. All media were purchased from Merck (Darmstadt, Germany).

ii. Liquid media

Nutrient broth was prepared by adding 8.0 g of nutrient broth to 1000 mL of demineralized water in a container and sterilized for 20 minutes at 120 °C. The media was purchased from Merck (Darmstadt, Germany).

3.1.4 Hydrocarbons

Three types of hydrocarbons were used in this study; used car oil was obtained from local gas station. Two types of crude oil (Miri and Masila blend) were provided by the Faculty of Chemical Engineering (UMP), and their physical properties are listed in (Table 3.1).

Table 3.1: Physical properties of Miri and Masila crude oil

Crude oil type	API gravity	Flash point	Density
Miri crude oil	32.6 degree	-11 °C	0.8714 kg/l
Masila crude oil	30.5 degree	< 25 °C	0.8691Kg/l

3.1.5 Microorganisms and their Isolation

Microorganisms used in all experiments were isolated by selective enrichment technique from Malaysian regions obtained from December 2010 until April 2011.

According to Ilyina et al. (2003), microorganism's isolation was carried out using selective medium (agar containing Miri crude oil, benzene and used car oil) as carbon source. One gram of contaminated soil sample was suspended in 10 mL of demineralized sterilized water. Sample was serially diluted by adding 1 mL of the mixture to 9 mL of demineralized sterilized water, solution was well mixed and procedure was repeated to the dilution 10^{-12}, 1.0 mL of each dilution was added to the media, distributed by spread technique and incubated at 27 °C for 21 days. The biggest colonies were chosen after seven days, 14 days and 21 days, inoculated on agar media and incubated at 27 °C for seven days to allow sufficient time for microbial growth. Pure stocks were prepared for the pure colonies for further experiments and identification stage later. The chosen single colonies were restreaked on agar plate and incubated for seven days at 27 °C. This procedure was repeated several times until pure colony was developed. The purity was confirmed under microscope when all the microbial cells had same Gram staining and morphological structure. Then using sterile loop, pure colonies were transferred into a tube or bottle of fresh agar medium (pure stock culture). The pure isolated microorganisms were named according to the regions: group K for Kuala Lumpur, group A for Selangor, group Z for Kajang, group M for Rawang and group S for Serdang.

3.1.6 Identification of Microorganisms

The microorganisms were identified according to the general principles of microbial classification, using Gram staining (Appendix A), biochemical test, selective media and macro- and microscopic examination of morphological characters (Sharma, 2007).

Isolated strains from contaminated soil were Gram stained in the lab, then a pure culture was sent to be identified in the Veterinary Laboratory, Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine (UPM) according to Bergey and Breed (1957) manual for bacteria identification. For fungi identification, it was conducted according to Gerlach and Nirenberg (1982), using Synthetic nutrient agar - SNA (Nirenberg, 1989) and PDA media by the microculture technique of Rivalier and Seydel (1932) modified method. Two strains were further identified to the genus level using BBL Crystal™ Identification Systems (Appendix B).

3.1.7 Preparation of Microbial Mixtures

In Table 3.2, the microbial mixtures were prepared based on the area of isolation. Some of the mixtures were prepared by mixing the isolated microbes that were adapted to live together in the same car workshops for a long time; for example, Kmix (from Kuala Lumpur), Amix (from Selangor), Zmix (from Kajang), Mmix (from Rawang) and Smix (from Serdang). The other way of mixing was by adding the microbial isolates from different car workshops together in the same mixture; for example, MS refereeing to the microbes isolated from Rawang and Serdang mixed together. Finally, there was one mixture which includes microbial isolates from all car workshops mixed together (Mix). (For more details, please refer Appendix C).

3.2 HYDROCARBON BIODEGRADATION SCREENING

According to the modified procedure of Lemos et al. (2002), qualitative determination of the degradation capacity of crude oils and used car oil of the isolated microbial strains (single and mixtures), was driven in 24 well cell culture clusters - flat

bottom with lid in polystyrene. The isolated microorganisms were inoculated in nutrient broth in separate tubes and allowed to grow for seven days at 27 °C.

Into each well, 1600 µL of sterilized nutrient broth, 200 µL of inoculated nutrient broth tubes and 10 µL of used car oil, Miri and Masila crude oils were added, individually. One well in each group was taken as a control (sterilized nutrient broth, used car oil or crude oils) without microbial addition. Other wells were used for microbial mixtures (Mix), where 22 isolated microbial strains were inoculated in nutrient broth and added to the well. The culture clusters were covered with polystyrene lid and incubated at 27 °C, results were recorded at zero time, after three days and then each seven days until 30 days. In the same way, microbial mixtures were prepared (Table 3.2) and added into different labeled wells, one well was taken as a control for each group alone. The control well did not contain any added microbial.

Table 3.2: Microbial mixtures for screening of oil samples degradation

Group name	**Content**
A mix	A1, A2, A3, A5, A6, A9, A15
K mix	K1, K3, K5, K11, K12
M mix	M1, M4, M7, M8, M9
S mix	S1, S4
Z mix	Z1, Z4, Z13
MS	M mix + S mix
ZS	Z mix + S mix
KA	K mix + A mix
AM	A mix + M mix
AZ	A mix + Z mix
AS	A mix + S mix
ZK	Z mix + K mix
MK	M mix + K mix
MZ	M mix + Z mix
SK	S mix + K mix
AK	A mix + K mix

- Microbial mixtures groups: K (from Kuala Lumpur), A (from Selangor), Z (from Kajang), M (from Rawang) and S (from Serdang) car workshops

3.3 MEASUREMENT OF BIODEGRADATION RATE BY ISOLATED MICROORGANISMS

3.3.1 Soil Preparation

According to the modified method of Ilyina et al. (2003), soil was collected from Universiti Putra Malaysia (UPM), autoclaved at 120 °C, for one hour to ensure all microbial flora were completely removed and to avoid its interference with the results.

In petri dishes, 50 g of sterilized soil was added. There were three groups of petri dishes. The first group was used car oil, the second group was for Masila crude oil, and the last group was for Miri crude oil. Each hydrocarbon (3% w/w) was added to the soil in the petri dishes and mixed thoroughly. Total petri dishes numbers were 24 for each group, according to the number of isolated strains and their mixtures in addition to one petri dish as a control (sterilized soil and crude oils or used car oil) without added microbial.

3.3.2 Microorganism Preparation

According to Ilyina et al. (2003), isolated microorganisms were inoculated separately in tubes containing 12 mL of nutrient broth, incubated at 27 °C for seven days. After seven days, 2 mL of each microbial culture were added to 10 mL of sterilized demonized water and added directly to each prepared petri dish as mentioned above. For microbial mixture preparation (Mix), 100µL of each strain solution of the 22 strains were added together in one tube, 10 mL of sterilized demonized water was added to the mixture. The mixture was added to the soil in petri dish. The control group did not contain any added microbial (sterilized soil, crude oils and used car oil, separately).

3.3.3 Measurement of Total Extractable Hydrocarbon Content

At zero time, 1 g of soil in control petri dish was weighed and transferred to dry, clean test tubes. Into this was added 10 mL of toluene, vortexed well and allowed to settle down for 30 min. The toluene-oil extracts were placed in cuvette wells and its absorbance was determined using spectrophotometer at 340 nm for used car oil and 360

for both Miri and Masila crude oil. The wave length was chosen after screening of several dilutions of crude oils and used car oil in the spectrophotometer. The best absorbance was at 340 nm and 360 nm for used car and crude oil, respectively. Then, all petri dishes were incubated at 27 °C for 30 days and aerated every seven days.

After 30 days, absorbance was taken again for each petri dish. Absorbance was converted to concentration by comparing it with standard curve. A calibration curve was obtained by measuring absorbance of dilute standard solution of all used hydrocarbons separately (Appendices D, E and F). Total hydrocarbon content was calculated after reading the absorbance of extracts from the spectrophotometer, exploiting from calibration curve and multiplying by an appropriate dilution factor (Osuji et al., 2006).

3.4 STUDY OF THE EFFECT OF AERATION, ADDING NPK AND MICROBIAL DEGRADERS ON HYDROCARBON BIODEGRADATION PROCESS

This experiment, aimed to find the best treatment method for laboratory contaminated soil with used car oil and Masila crude oil by studying the effect of aeration adding NPK. This experiment also looked at the effect of added microbial biodegraders isolated from contaminated car workshops soil on the biodegradation process and indigenous microbes.

3.4.1 Soil Sample Collection and Preparation

Soil was collected from Universiti Putra Malaysia (UPM) and cleaned from stones and other impurities. Soil samples were divided into two groups, each group contained five containers (20 cm x 20 cm x 15 cm), and in each container, 700 g of soil was distributed according to the method of Ayotamuno et al. (2006).

3.4.2 Experimental Design

Used car oil (200 mL) was added to the first group. Masila crude oil (200 mL) was added to the second group. In each group, the control container contained soil and oil samples only without any added materials. Container (1) contained soil, oil samples

and the soil was tilled twice a week by shovel to provide the necessary aeration (Chorom et al., 2010). Container (2) contained soil, oil samples and NPK 1:1:1 (25 g) (Ubochi et al. 2006). Container (3) contained soil, oil samples and a mixture of strains which were isolated earlier. Adding the microbial was done by inoculating 22 isolated microbial strains that found to be crude oil and used car oil biodegraders in 50 mL of nutrient broth, incubated for seven days at 27 °C, then 25 mL of nutrient broth was mixed with 75 mL of sterilized deionized water and 100 mL of the mixture was added. The last group (Container 4) contained soil, oil samples and NPK 1:1:1 (25 g) with aeration done twice a week in addition to the microbial mixture, which was done similarly as mentioned above (Fig 3.1).

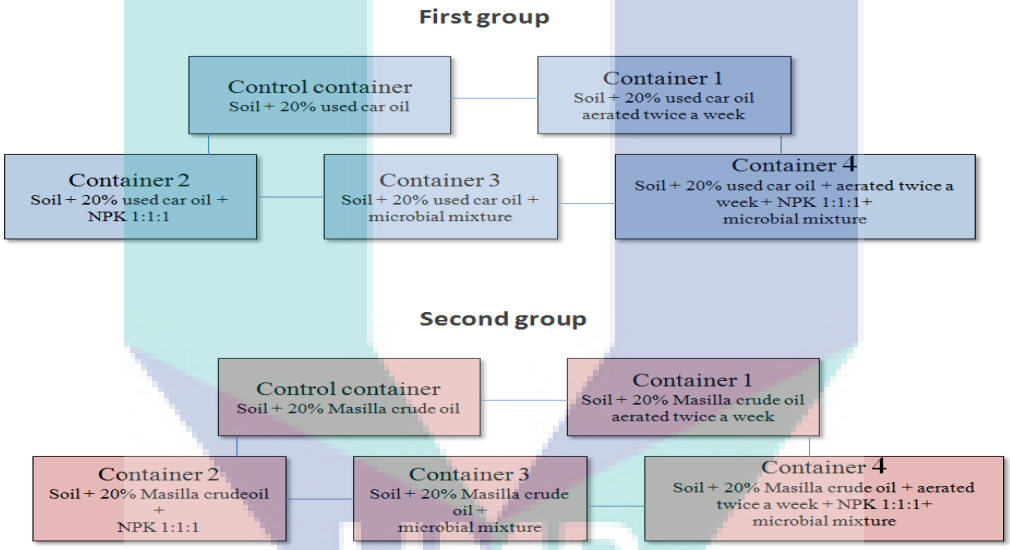


Chart 3.1 Experimental design for the effect of aeration, NPK and microbial biodegraders adding on the biodegradation process and indigenous microbes.

All containers were left under a shade, at temperature ranging from 20-30 °C, protected from rain and humidity maintained everyday by spraying with distilled sterilized water to avoid dryness. The containers were left for three days to allow evaporation of the oil samples by normal ways and to avoid interference with losses that might happen in the experiment. Samples were analyzed at zero time, after three days then every seven days for 42 days. The analysis was to determine pH, CFU of total heterotrophic microbes and hydrocarbon utilizing microbes in addition to the measurement of percentage of degradation of hydrocarbon.

3.4.3 Total Heterotrophic Microbial Count

Soil (1 g) was weighed and diluted with 10 mL of distilled and sterilized water. The mixture was serially diluted until 10^{-9}, 100 µL of each dilution was spread on nutrient agar plate and incubated for 24-48 hrs at 27 °C (Ubochi et al., 2006). The total count of all microorganisms was determined. The procedure was done at zero time, after three days then every seven days for 42 days. Colony Forming Unit (CFU) was determined as follow.

CFU/standard unit volume = number of colonies x dilution factor x standard unit volume/aliquot plated

3.4.4 Hydrocarbon Utilizing Microorganisms

From each of the above mentioned dilution mixtures, 100 µL were spread on mineral salt media. Sterilized filter paper (70 mm diameter) was soaked in oil samples (crude oil and used car oil, separately) and placed on the cover of the petri dishes. The medium was inverted on the cover and incubated for seven days at 27 °C. The hydrocarbon degrading microorganisms were determined. The procedure was done after three days and each seven days for 42 days, and CFU was also determined, according to Ubochi et al. (2006) method.

3.4.5 Calculation of Percentage of Oil Samples Degradation

The percentage of degradation of crude oil and used car oil was calculated by comparing the concentration results of the test with those of the control using the formula:

$$\text{Percentage degradation of oil samples} = \frac{(\text{Concentration of control} - \text{Concentration of test})}{\text{Concentration of control}} x\ 100$$

3.4.6 Measurement of Percentage of Degradation of Oil Samples

At zero time 1 g of soil in control container was weighed and transferred to dry, clean test tubes. Into this was added 10 mL of toluene, vortexed well and allowed to settle down for 30 min. The toluene-oil extracts were placed in cuvettes and its absorbance was determined by using spectrophotometer at 340 nm for used car oil and 360 for Masila crude oil. The method was adopted from Osuji et al., (2006). The procedure was conducted at zero time, then every seven days for 42 days. Absorbance was taken again for each container. Absorbance was converted to concentration by comparing it with standard curve.

3.4.7 Soil pH changes during the period of study

With each sampling for soil hydrocarbon content determination, 1 g of soil was weighed in a clean glass beaker and 10 mL of distilled water was added. The mixture was stirred to obtain soil slurry. The sample was left to stand for 30 minutes. Using standardized pH meter at pH 4.0 and 7.0, soil pH was measured (Ebere et al., 2011) to determine the pH changes that occurred during the period of the study.

3.4.8 Statistical Analysis

The statistical analysis was performed using IBM SPSS Statistics 19 software. Each experiment was performed in triplicate. Results were expressed as the means, and standard deviation (± S.D.). Statistical analysis was performed using ANOVA followed by Tukey and Dunnet tests. The statistical significance was expressed as *, $p < 0.05$. (Please refer to Appendix G for the statistical analysis).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 ISOLATION OF MICROBIAL STRAINS

Five contaminated soil samples were collected from five car workshops in Kuala Lumpur region (Kuala Lumpur, Selangor, Kajang, Rawang and Serdang), 22 microbial strains were isolated and classified depending on their macro- and microscopic features and Gram staining results. Their cultivation was performed on different media (Table 4.1).

Table 4.1: Growth of microbial isolates from different areas around Kuala Lumpur on media with different carbon sources

Area	**Selective media**			
	Agar	**Agar + benzene**	**Agar + used car oil**	**Agar + crude oil**
Kuala Lumpur	K11, K3. K5	K1	K12	-
Selangor	A1	-	A6, A9, A3, A15	A5, A2
Kajang	-	Z1, Z13	Z4	-
Rawang	-	M1, M7	M8, M9, M4	-
Serdang	-	S1, S4	-	-

It was found from the results that the microbial growth occurred mostly on agar media mixed with benzene and used car oil, showing that they were the most preferable hydrocarbon source by microorganisms. This was suggested due to the presence of these hydrocarbons in the environment that the microbes were isolated from. The least growth

was in agar media mixed with crude oil, due to the complex hydrocarbons that the microbes needed longer time to degrade it.

4.1.1 Identification of Isolated Microorganisms

i. Gram staining

Twenty two strains were isolated from contaminated soil collected from five car workshops around Kuala Lumpur. The strains were Gram stained as a first step in the identification stage. The macro- and microscopic features were also recorded.

ii. Selective media and biochemical test based identification

The pure isolated microbial strains were inoculated on agar plate and incubated at 27 °C for seven days. Biochemical test, selective media and macro- and microscopic examination of morphological characters were performed in the Veterinary Laboratory, Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine (UPM) by bacteriologist Prof. Dr. Saleha Abdul Aziz (UPM).

According to the results shown in Table 4.2, 19 strains were identified at the genus levels, one to the species level and three were just identified morphologically. The laboratory result is shown in Appendix H. The result shows that the microbial strains were a mixture of bacteria, fungi and yeast which were adapted to live in the contaminated soil.

Table 4.2: Result of identification of the isolated microbial strains

Code	Result
A1	*Achrombacter* spp.
A2	*Aeromonas* spp.
A3	*Acinetobacter* spp. *Klebsiella pneumoniae*
A5	*Pseudomonas* spp.
A6	*Corynebacterium* spp.
A9	*Corynebacterium* spp.
A15	*Corynebacterium* spp.
K1	*Pseudomonas* spp.
K3	*Penicillium* spp.
K5	*Corynebacterium* spp.
K11*	*Enterobacteriaceae* (*Enteric rods*)
K12	*Corynebacterium* spp.
M1	*Streptomyces* spp.
M4	*Corynebacterium* spp.
M7	Budding yeast cells
M8	*Streptomyces* spp.
M9	*Cladosporum* spp.
S1*	*Actinobacillus*
S4	*Geotrichum* spp.
Z1	*Corynebacterium* spp.
Z4	*Corynebacterium* spp.
Z13	*Corynebacterium* spp.

* indicates that these strains were identified using biochemical kits (PPL crystal identification system Appendix B).

4.1.2 Hydrocarbon Biodegradation Screening for Isolated Single Strains

Microbial biodegradation for different oil samples were performed visually for 30 days, by inoculating isolated microorganisms in cell culture cluster wells containing nutrient broth. Oil samples were then added to each well. Microbial mixtures were prepared to observe the effect of many microbes together in order to find the best microbial strain or microbial blend which can degrade the oil samples to the greatest extent and using the result as a primary indication for the biodegradation ability of microbial strains and microbial mixture. The results were recorded at zero time, after three days then every seven days for 30 days.

Plate 4.1, shows the result of biodegradation screening of isolated microorganisms on used car oil. As the result shown, at zero time the used car oil was distributed evenly on the surface. After three days, the distribution pattern changed into ring like on the borders of the wells. The microbial consumption of used car oil (biodegradation) took place starting from the day 14. After 30 days, the change in the used car oil amount and morphological appearance was clear. Generally, most of the isolated strains showed effect on used car oil biodegradation. Some microbial strains degraded the used car oil to a higher extent than the other strains such as A1, A15, K1 and M8 which were identified as *Achrombacter* spp, *Corynebacterium* spp, *Pseudomonas* spp. and *Streptomyces* spp, respectively compared to the control which did not contain any microbial species.

The least degrading isolated strains for used car oil were A2 and M7 which were identified as *Aeromonas* spp. and Budding yeast cells, respectively compared to the control which did not contain any microbial species.

The results of biodegradation screening of isolated microorganisms on Miri crude oil are shown in plate 4.2 below. The biodegradation of Miri crude oil was most clear at the day 30. The most clear degrading strains compared with the control were A1, A9 and Z13 which were identified as *Achrombacter* spp and *Corynebacterium* spp., respectively. While the least degrading strains were A2 and S4 which were identified as *Aeromonas* spp. and *Geotrichum* spp., respectively.

Finally, the biodegradation screening result of Masila crude oil is shown in plate 4.3. The biodegradation effect was not as high as used car oil and Miri crude oil. This might be due to high concentration of toxic compounds in the crude oil especially the volatile compounds. This suggestion was supported by Zhang et al. (2006) finding, where there were various toxic effects of crude oil residues on the growth and reproduction of soil bacteria. Some of the isolated strains produced emulsion in a form of white mass which was an indication for the dissolving of the oil in the media. The best degrading strains were A9 and the strains mixture. A9 was identified as *Corynebacterium* spp. while the least degrading strains were S1 and K1 which were identified as *Actinobacillus* and *Pseudomonas* species.

In general, it was found from the screening results that the microbial degradation screening for the used car oil was the most apparent morphologically, where there were changes in oil amount and distribution on the surface compared with the control. This, suggest that the original sampling environmental isolation condition, which was the contamination of the sampling areas mostly with the used engine oil, was a very important factor in this matter. The microorganisms were adapted to these conditions and with time, they started using this hydrocarbon as a carbon and energy source. There was also a biodegradation effect on Miri and Masila crude oil. This was due to the presence of some hydrocarbons that can also be found in the used car oil, but the biodegradation extent was less because of the presence of the toxic volatile materials (Zhang et al., 2006), particularly in the Masila crude oil, which affected the microbial growth pattern.

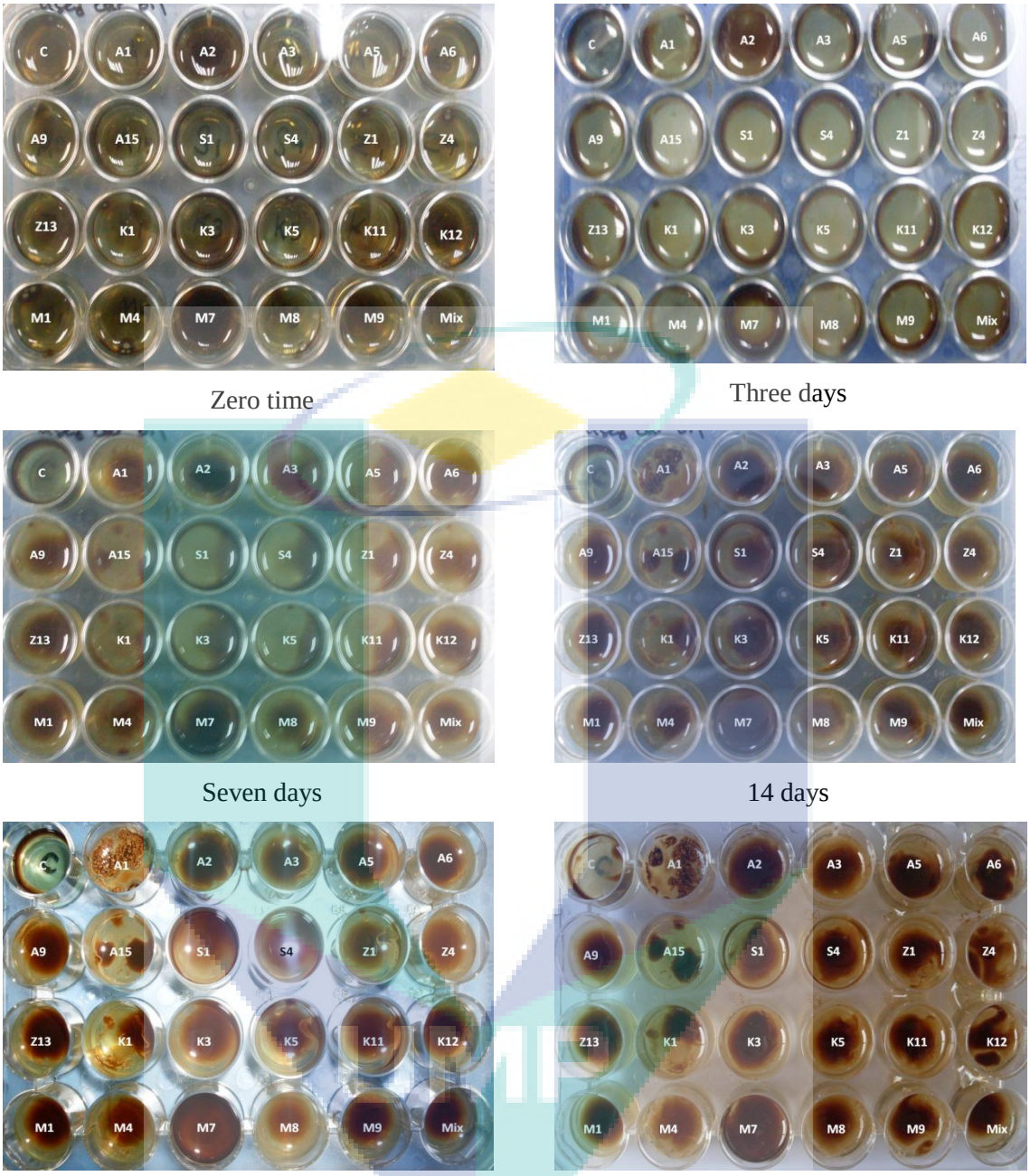


Plate 1. Used car oil biodegradation screening result; C is control. A1 (*Achrombacter)*, A2 (*Aeromonas)*, A3 (*Acinetobacter)*, A5 (*Pseudomonas)*, A6 (*Corynebacterium)*, A9 (*Corynebacterium)*, A15 (*Corynebacterium)*, K1 (*Pseudomonas)*, K3 (*Penicillium*), K5 *(Corynebacterium*), K11 (*Enterobacteriaceae*), K12 (*Corynebacterium*), M1 (*Streptomyces*), M4 (*Corynebacterium*), M7 (yeast cells), M8 (*Streptomyces*), M9 (*Cladosporum*), S1 (*Actinobacillus*), S4 (*Geotrichum* spp). Mix is a mixture of all isolated strains

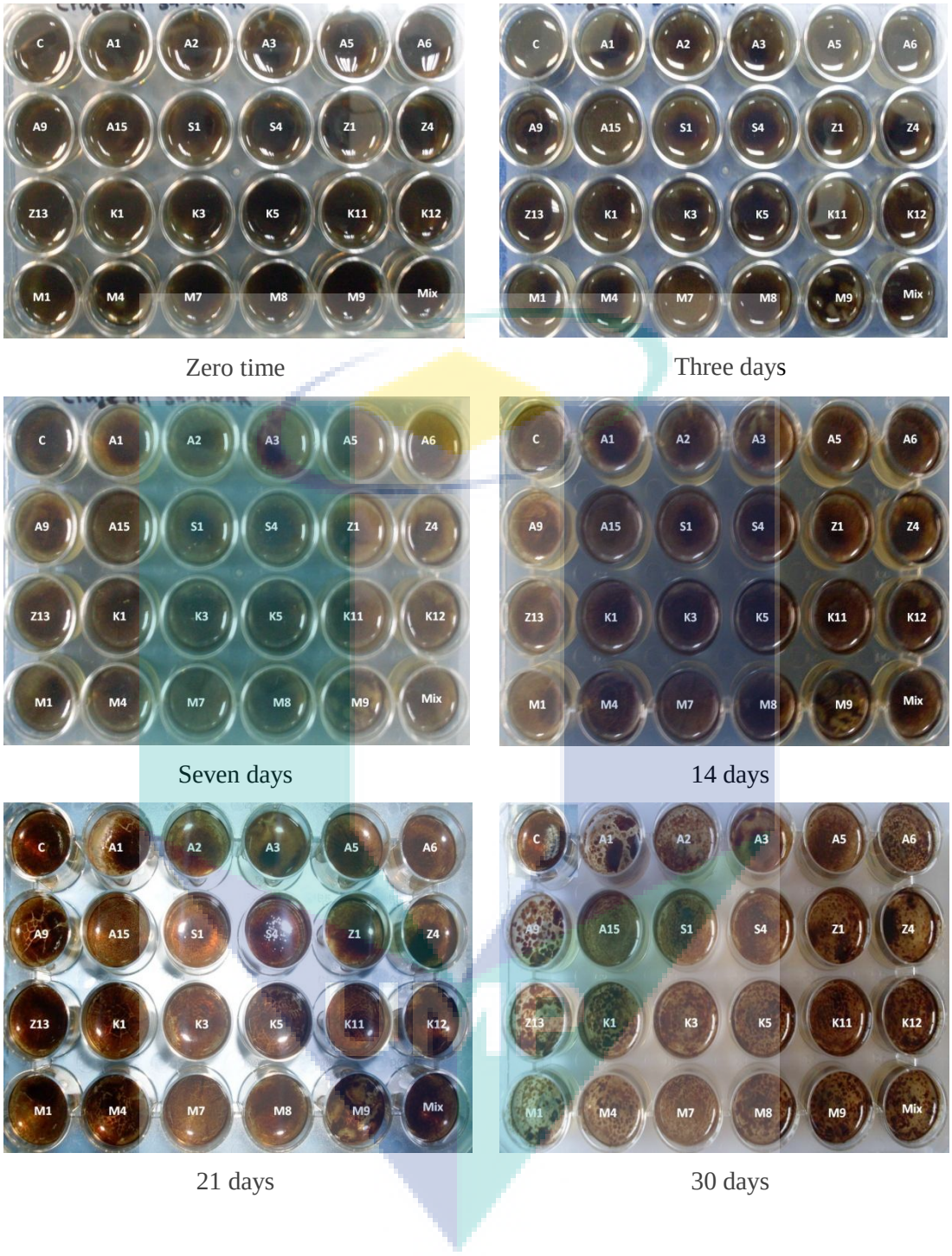


Plate 2. Miri crude oil biodegradation screening result; C is control. A1 (*Achrombacter)*, A2 (*Aeromonas)*, A3 (*Acinetobacter)*, A5 (*Pseudomonas)*, A6 (*Corynebacterium)*, A9 (*Corynebacterium)*, A15 (*Corynebacterium)*, K1 (*Pseudomonas)*, K3 (*Penicillium*), K5 *(Corynebacterium*), K11 (*Enterobacteriaceae*), K12 (*Corynebacterium*), M1 (*Streptomyces*), M4 (*Corynebacterium*), M7 (yeast cells), M8 (*Streptomyces*), M9 (*Cladosporum*), S1 (*Actinobacillus*), S4 (*Geotrichum* spp). Mix is a mixture of all isolated strains.

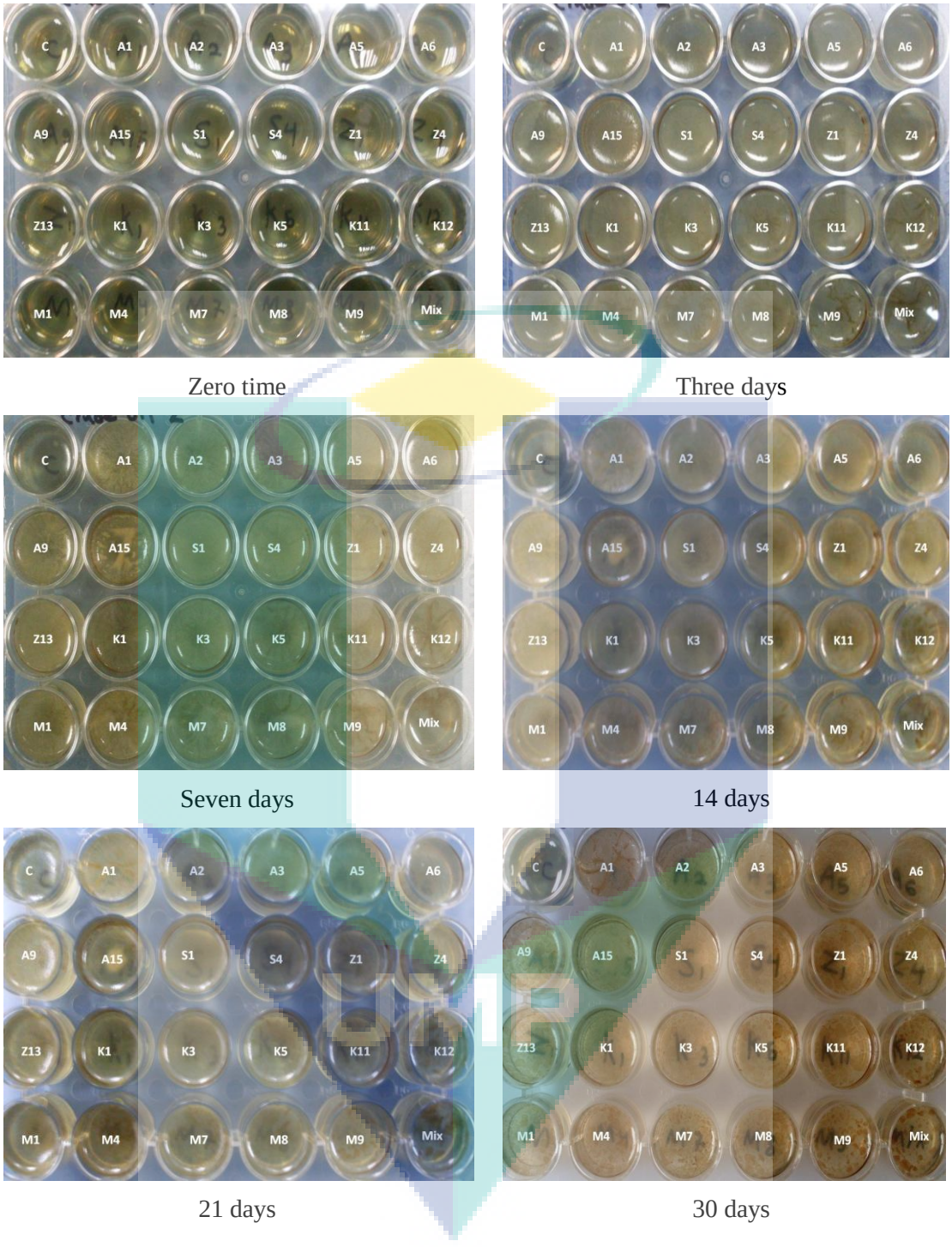


Plate 3. Masila crude oil biodegradation screening result; C is control .A1 (*Achrombacter)*, A2 (*Aeromonas)*, A3 (*Acinetobacter)*, A5 (*Pseudomonas)*, A6 (*Corynebacterium)*, A9 (*Corynebacterium)*, A15 (*Corynebacterium)*, K1 (*Pseudomonas)*, K3 (*Penicillium*), K5 *(Corynebacterium*), K11 (*Enterobacteriaceae*), K12 (*Corynebacterium*), M1 (*Streptomyces*), M4 (*Corynebacterium*), M7 (yeast cells), M8 (*Streptomyces*), M9 (*Cladosporum*), S1 (*Actinobacillus*), S4 (*Geotrichum* spp). Mix is a mixture of all isolated strains.

4.1.3 Hydrocarbon Biodegradation Screening for Isolated Microbial mixture

In order to evaluate the microbial mixture efficacy to degrade crude oil and used car oil compared to a single strain, sterilized nutrient broth was added into labeled cell culture cluster - flat bottom with lid in polystyrene into each well, and inoculated nutrient broth with a mixture of microbial strains in random pattern (Table 3.2) were added to the wells separately. One well in each group was taken as control (nutrient broth and used car oil or crude oil only with no microbes added). Finally, used car oil, Miri and Masila crude oil were added to all wells according to the labeled groups. Plates were covered with polystyrene lid and incubated for 30 days. Results were recorded at zero time, after three days then every seven days for 30 days (Plate 4).

According to the results, generally, the microbial mixtures showed degradation of oil samples. The biodegradation effect was more apparent on the used car oil, where the microbial mixtures Amix, MS and SA (Table 3.2) showed the highest changes in the amount and morphological appearance of the used car oil compared with the control well which did not contain any added microbes.

At the end of the study, the microbial mixture, Amix, MS, SA and ZM showed the most apparent changes in used car oil, the mixtures MS and MK in Miri crude oil and MS, MK, and the mixtures ZM in Masila crude oil.

On the other hand, the biodegradation effect by microbial mixtures took place also in Miri crude oil, as well as Masila crude oil (Plate 4). Generally, the biodegradation effect of all microbial mixtures was apparent. Studies have shown that a single microbial species can degrade only one or two classes of hydrocarbon within a crude oil. Mixtures of microorganisms are required to significantly biodegrade a large fraction of crude oil (Leila et al., 2006).

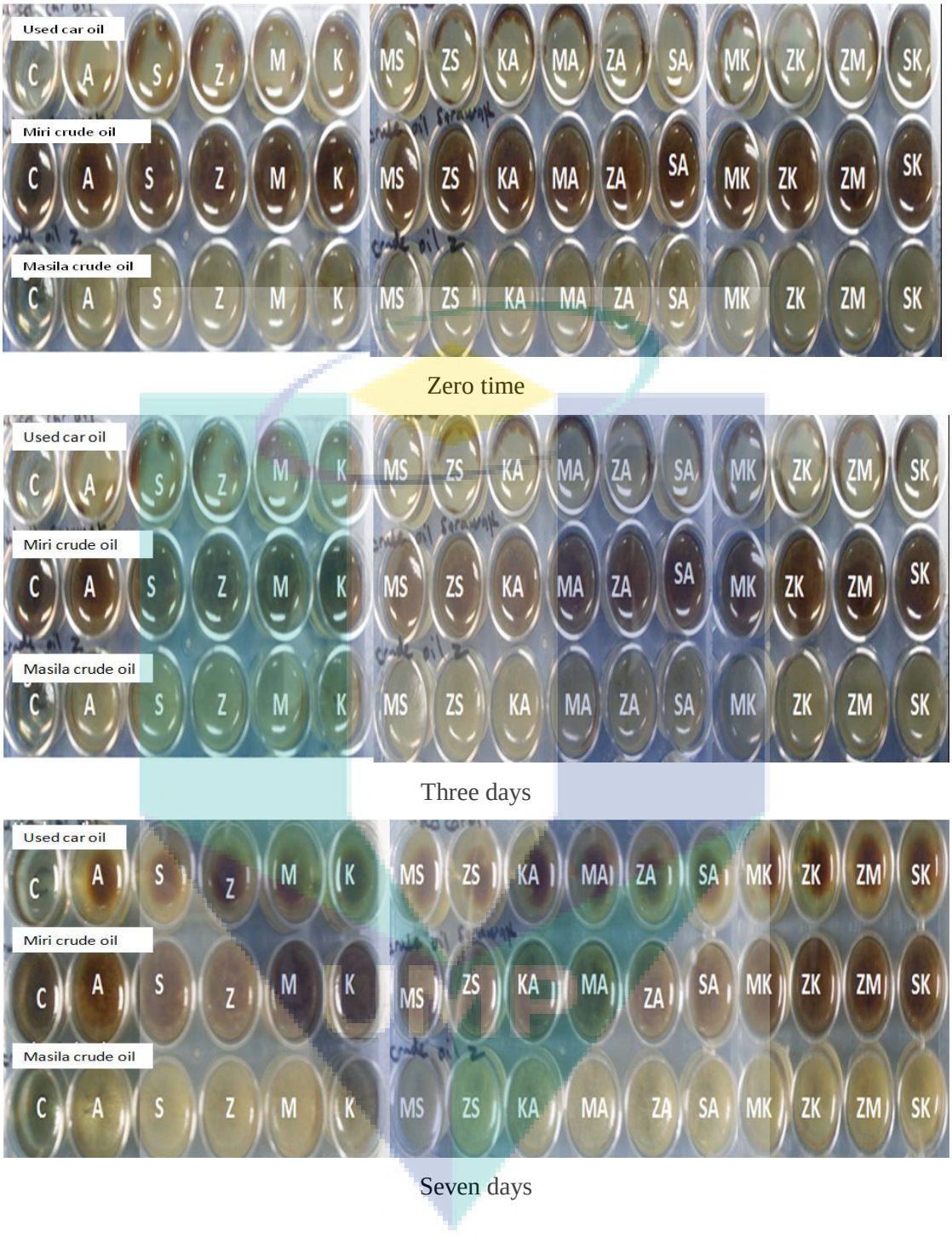
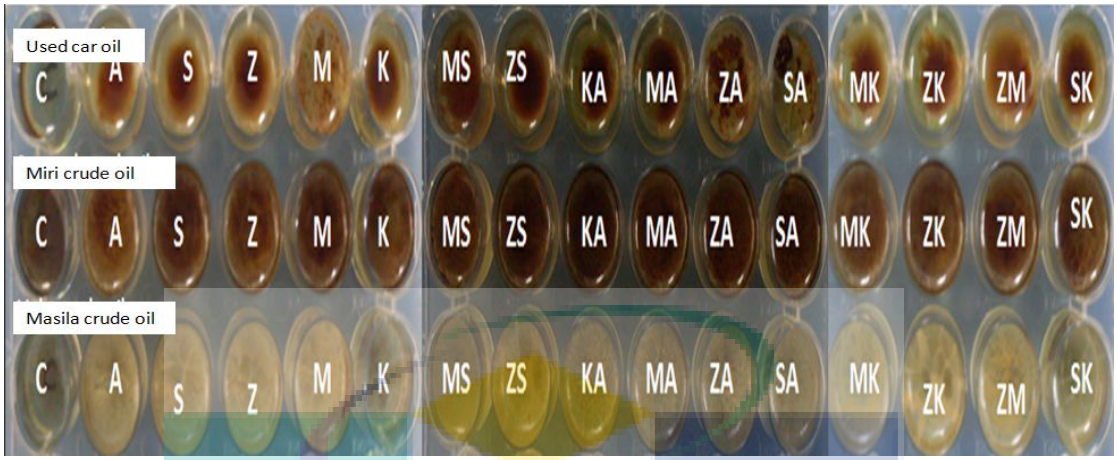
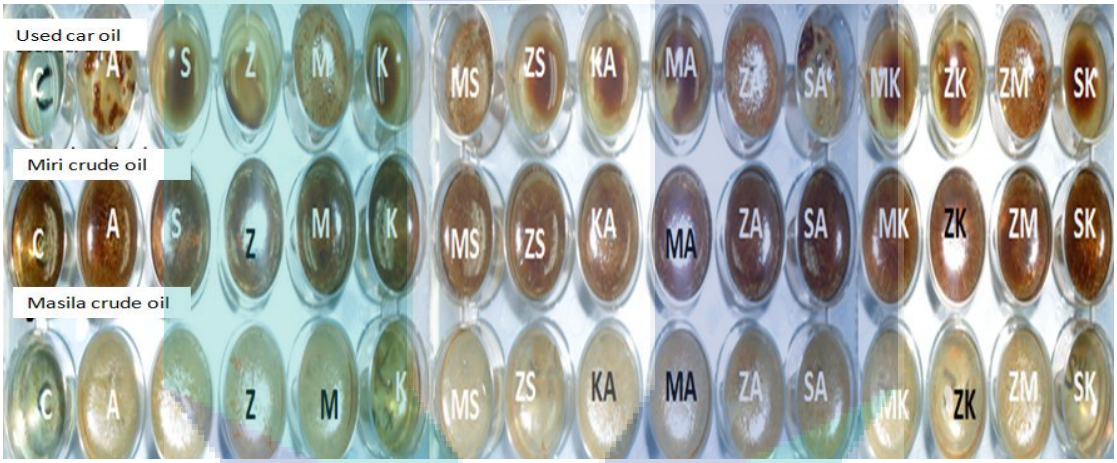


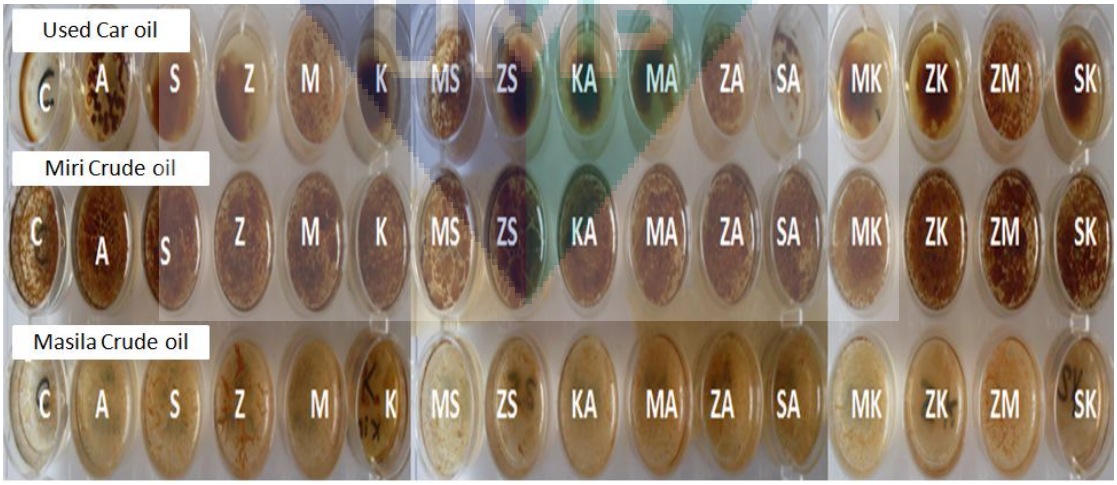
Plate 4. Used car oil, Miri and Masila crude oil biodegradation screening result by microbial strains mixtures; K (from Kuala Lumpur), A (from Selangor), Z (from Kajang), M (from Rawang) and S (from Serdang). C is control.



14 days



21 days



30 days

Plate 4. Continued

4.2 THE RATE OF BIODEGRADATION OF OIL SAMPLES

4.2.1. Used car oil

Used car oil content of the soil samples was determined by toluene cold extraction by adding toluene to the soil sample. The liquid phase of the extract was measured at 340 nm using spectrophotometer. The total petroleum hydrocarbon (TPH) in soil was estimated with reference to standard curve, which was derived from fresh used engine oil diluted with toluene. The used car oil biodegradation was tested using single isolated strains as well as microbial mixtures (Table 4.3). In general, the result for single strains biodegradation (Figure 4.1) showed that the percentages of oil degradation were significantly different from the control, which did not have any added microbes. Strain Z4 which was identified as *Corynebacterium* spp. had the highest effect with 72.97 % of degradation, followed by strain M9 (*Cladosporum* spp.) with 69.42 % of degradation and M8 (*Streptomyces* spp.) with 68.9 % of degradation.

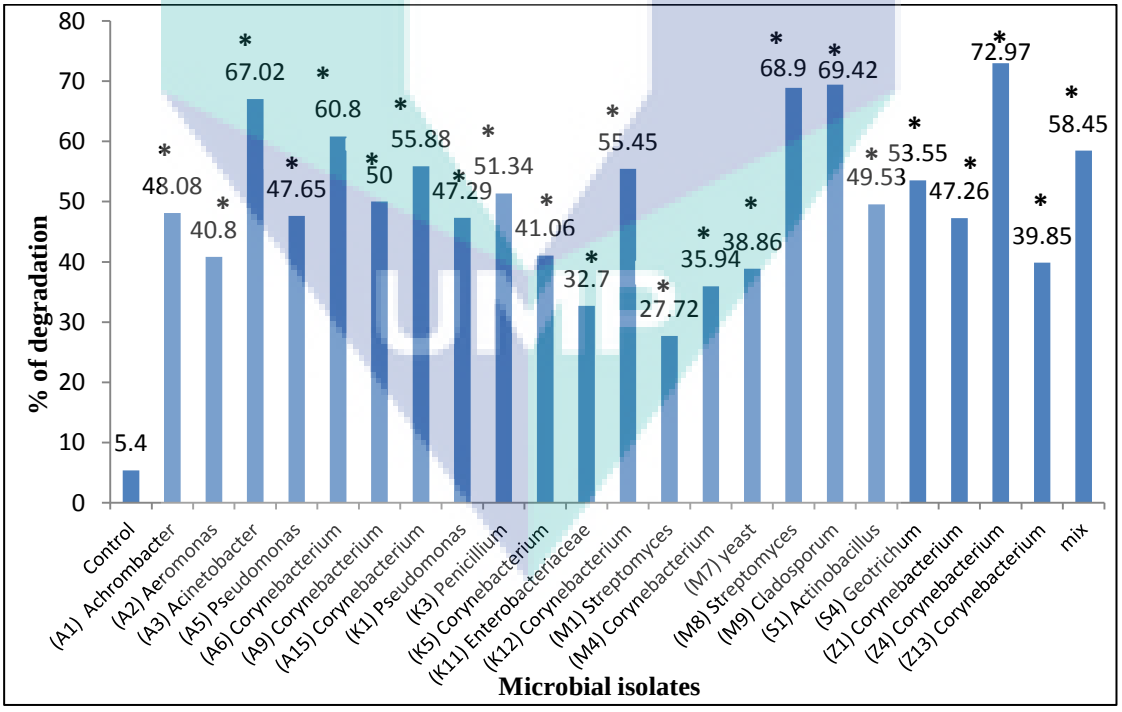


Figure 4.1 Used car oil microbial degradation rate for single isolated strains after 30 days. Significant difference *$P < 0.05$ compared with the control.

Strains M1 (*Streptomyces* spp.), K11 (*Enterobacteriaceae)* and M4 (*Corynebacterium* spp.) exhibited the least effects with 27.72 %, 32.7 % and 35.94 % of degradation, respectively. However, all of them were significantly different from the control.

The result of used car oil biodegradation by microbial mixtures (Figure 4.2) showed similar effects for single strains. Microbial mixture Zmix was highly effective with 72.49 % of degradation followed by microbial mixture Kmix and AZ with 71.32 % and 61.63 %, respectively. The high effect of microbial mixture Zmix was compatible with the highest single strains effect of Z4 (*Corynebacterium* spp.) due to its presence in the mixture, while Microbial mixture Kmix, which contained K1, K3, K5 and K12 (*Pseudomonas* spp., *Penicillium* spp. and *Corynebacterium* spp.) generally exhibited good effects. This suggested that they worked in synergistic effect on the biodegradation due to their original presence and growth in the same place from where they were isolated. The same was applied to microbial mixture Amix and Zmix strains which showed good effects when they were mixed together.

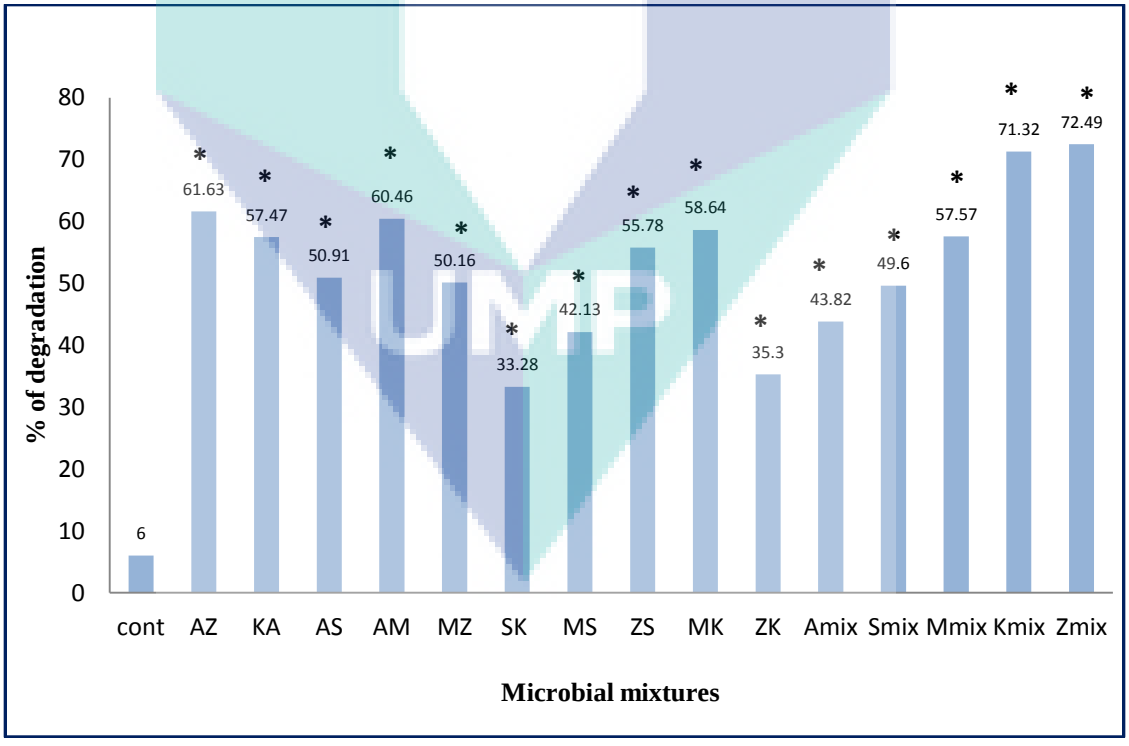


Figure 4.2 Used car oil microbial degradation rate for mixtures isolated strains after 30 days. Significant difference *$P < 0.05$ compared with the control

On the other hand, SK and ZK mixtures showed weak effect with 33.28 % and 35.3 % of degradation, respectively. This might be due to the microbial competition between those strains and toxin secretions. However, all the microbial mixtures showed significant difference compared with the control

Based on the results shown above, the microbial mixtures were more successful in used car oil biodegradation than the single strains, particularly those which have been isolated from the same car workshop and used together to prepare a mixture. This was because of the microbial adaption between them occurred for a long time in the same place and in the same environmental condition.

4.2.2 Miri crude oil

Miri crude oil content in the soil samples was determined by toluene cold extraction. The liquid phase of the extract was measured at 360 nm using spectrophotometer. The total petroleum hydrocarbon (TPH) in soil was estimated with reference to standard curve derived from Miri crude oil diluted with toluene. The Miri crude oil biodegradation was tested using single isolated strains as well as microbial mixtures (Table 3.2).

The result for Miri crude oil single strains biodegradation (Figure 4.3) showed that strain Z13 (*Corynebacterium* spp.) had 54.33% of degradation and A5 (*Pseudomonas* spp.) had 47.89% of degradation. Strains A2 (*Aeromonas* spp.), K1 (*Pseudomonas* spp.) and M1 (*Streptomyces* spp.) exhibited the least effects with 7.4%, 15.47% and 20.03% of degradation, respectively. A2 *(Aeromonas* spp.) did not show significant difference from the control. The well contained mixture of all isolated strains had the highest effect with 57.51% of degradation, which gave the indication that the microbial mixture was more effective in Miri crude oil degradation than single strains.

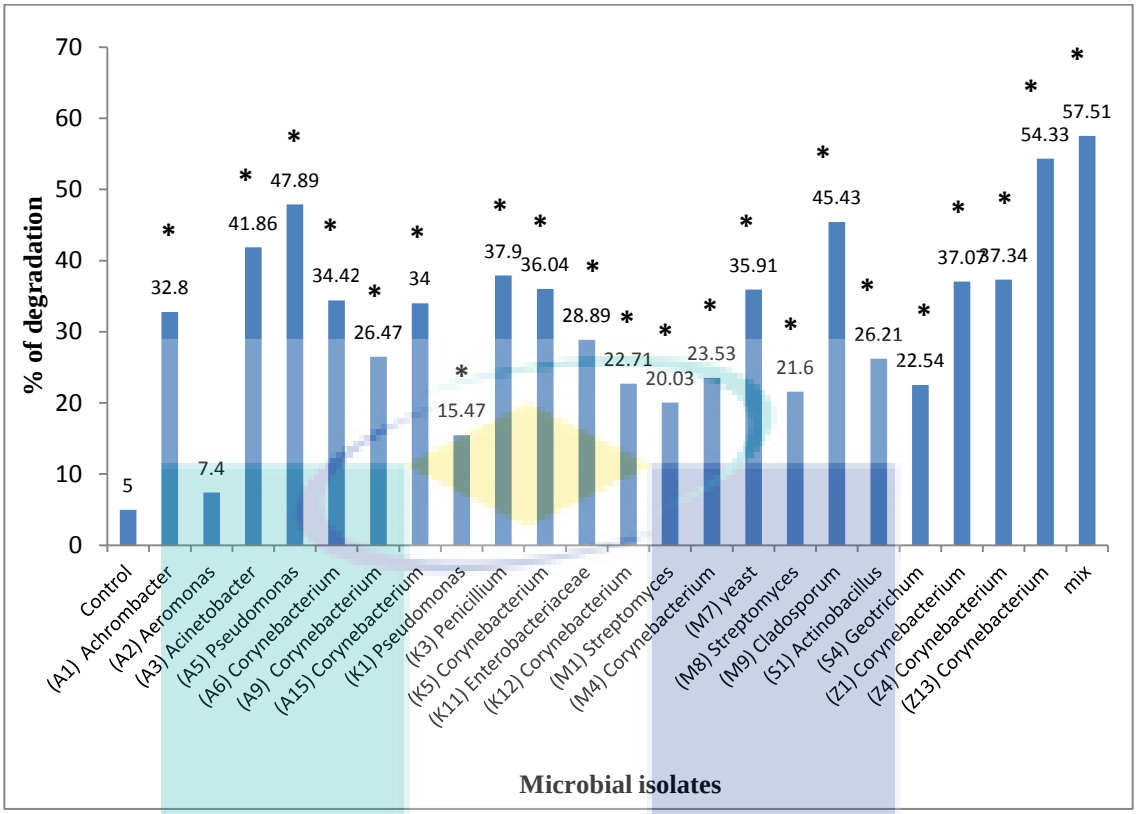


Figure 4.3 Miri crude oil microbial degradation rate for single isolated strains after 30 days. Significant difference *$P < 0.05$ compared with control

The result of Miri crude oil biodegradation by microbial mixture (Figure 4.4) showed much higher effects than single strains. Microbial mixture MS was highly effective with 84.61% of degradation followed by the mixtures AM and Kmix with 83.44 % and 82.34 % of degradation, respectively. The high effect of microbial mixture AM was due to the presence of A3 (*Klebsiella pneumonia*), A5 (*Pseudomonas* spp.) and M9 (*Cladosporum* spp.) in the mixture which was highly effective as single strains suggesting the possibility of synergistic effect. The same goes with AM mixture, while, the Kmix mixture which also exhibited good effect on the biodegradation of used car oil, exerted their effect due to their presence and growth in the same car workshop where originally they were isolated from. By contrast, Amix and Zmix mixtures showed less effect with 43.31 % and 51.29 % of degradation, respectively. However, their effects were significantly different from the control.

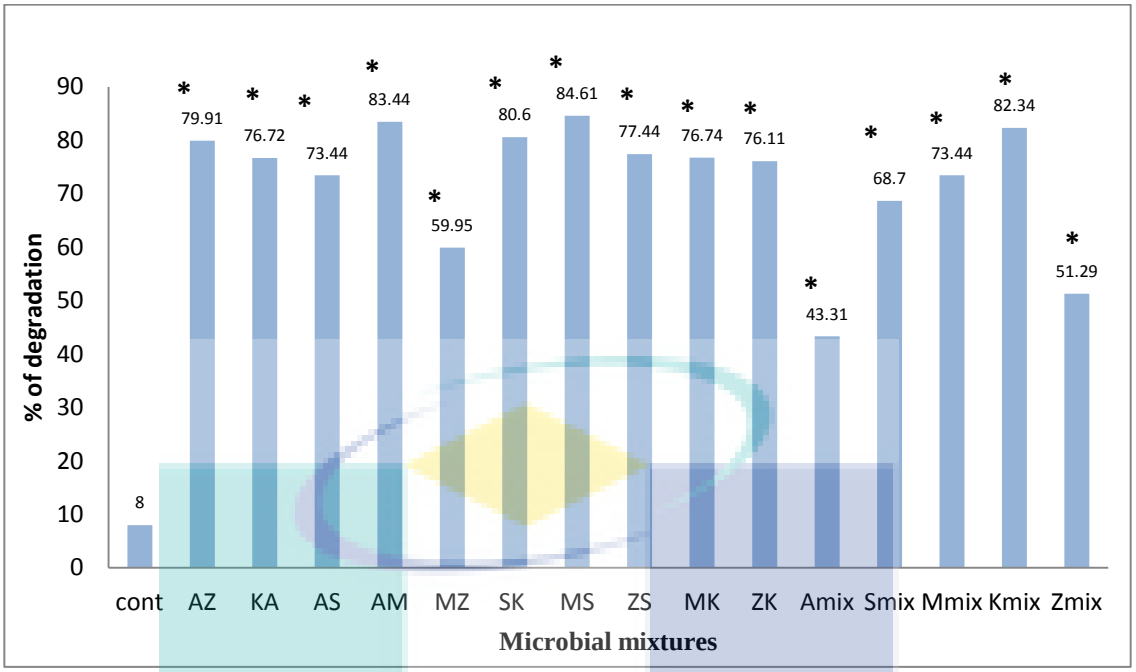


Figure 4.4 Miri crude oil microbial degradation rate for mixtures isolated strain after 30 days. Significant difference *$P < 0.05$ compared with control

From the results shown above, the microbial mixtures were much highly effective in Miri crude oil biodegradation than the single strains. This was due to the complex compound in the crude oil which needed more microbial species in order to manage to degrade it to more simple compounds.

4.2.3 Masila crude oil

The Masila crude oil biodegradation was tested using single isolated strains as well as microbial mixtures (Table 3.2).

The result for Masila crude oil single strains biodegradation (Figure 4.5) showed that all the microbial strains had weak effect on crude oil biodegradation. Strain A3 (*Klebsiella pneumonia*) showed the highest effect among the other strains with 33.81 % of degradation, while strain M7 (budding yeast cells) was the least effective with 4.6 % of degradation.

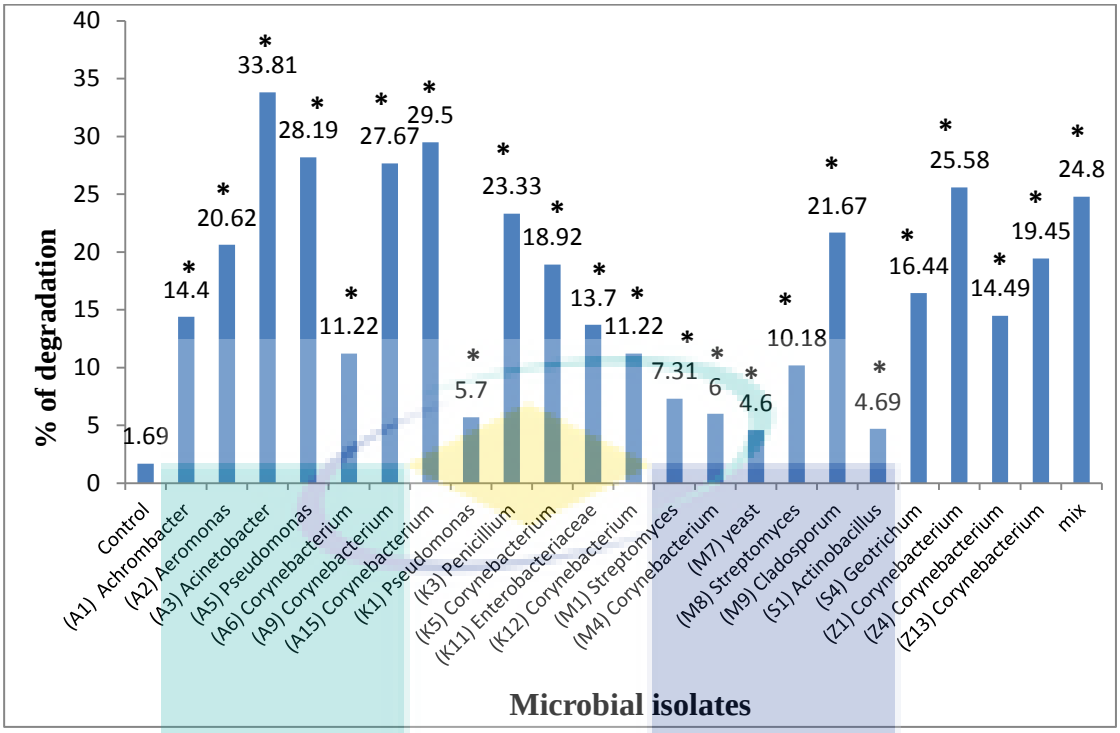


Figure 4.5 Masila crude oil microbial degradation rate for single isolated strains after 30 days. Significant difference $*P < 0.05$ compared with control

The result of Masila crude oil biodegradation by microbial mixtures (Figure 4.6) showed higher effects than single strains. Microbial mixture Smix was effective with 49.47 % of degradation followed by microbial mixture Mmix and Amix with 47.46 % and 46.19 % respectively. They also showed significant difference from control. On the other hand, microbial mixture ZK and SK were the least effective with 11.09 % and 12.15 % of degradation. This weak effect might be due to the microbial competition between those strains and toxin secretions (Zhang et al., 2006).

All in all, it can be concluded that of the isolated strains, the strain A1 (*Achrombacter* spp.) is the most effective in oil samples biodegradation. It degraded 48 % of used car oil, 32 % of Miri crude oil and 14 % of Masila crude oil after 30 days. This was compatible with Ghevariya et al. (2011) report, where multiple PAHs degrading halotolerant *Achromobacter xylosoxidans* was isolated from crude oil contaminated site, resulting in 40 % chrysene degradation after one week. *Achromobacter aerogenes* was also isolated from used engine oil polluted soil. Their

ability to biodegrade the used engine oil was evaluated, and it was found to be 80 % of the oil in three weeks (Adelowo et al., 2006).

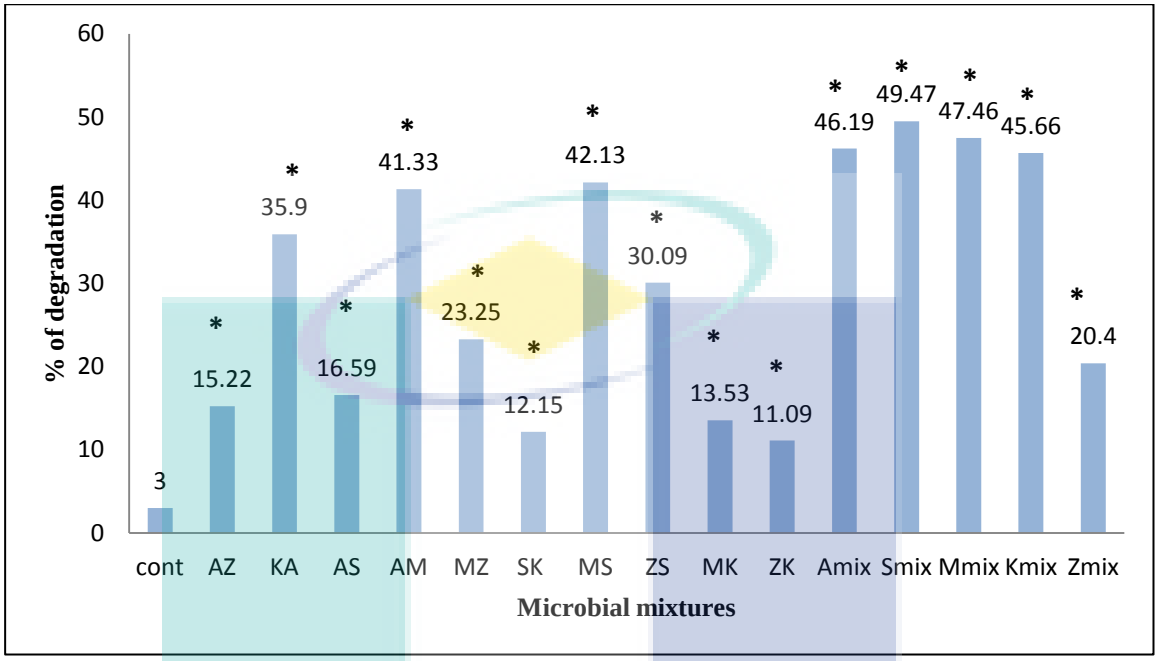


Figure 4.6 Masila crude oil microbial degradation rate for mixtures isolated strain after 30 days. Significant difference *$P < 0.05$ compared with control

This might illustrate the used car oil degradation by this species which contain some halogens resulted from engine oil combustion (Chung et al., 2007). The strains A5 and K1 (*Pseudomonas* spp.) were found to be different by macroscopic and microscopic characteristics. These strains degraded used car oil by 47.65 % and 47.29 %, respectively, degraded Miri crude oil by 48.89 % and 15.47 %, respectively, while the same strains degraded Masila crude oil by 28.19 % and 6.70 %, respectively. *Pseudomonas fragi* was also isolated from soils polluted with used motor oil. The organism utilized 73.30 % of the oil after three weeks (Adelowo et al., 2006).

Strain A2 (*Aeromonas* spp.) degraded used car oil, Miri and Masila crude oil by 40.80 %, 7.40 % and 20.62 %, respectively. A3 (*Acinetobacter* spp., *Klebsiella pneumonia*) degraded used car oil, Miri and Masila crude oil by 67.02 %, 41.86 % and 33.81 %, respectively. In a previous study, bacteria isolated from tropical areas around the Indian peninsula, showed high phosphates activity. The isolate was identified as *Klebsiella pneumonia*. It showed high adherence to undecane and hexadecane. The

degradation of crude oil by this isolate was 62 % (DeSouza et al., 1996). Bacterial species *Acinetobacter* was previously isolated from sediment and water of a crude oil contaminated river. *Acinetobacter calcoaceticus*, *Aeromonas* spp. and *Pseudomonas fluorescens* showed tolerance to varying concentrations of the oil (Diesel tolerant bacteria: DTB). *Aeromonas* spp. and *Pseudomonas*. *fluorescens* showed potential to degrade the diesel oil (Diesel-degrading bacteria: DDB) (Kayode-Isola et al., 2008). *Acinetobacter* (*Klebsiella*), and *Pseudomonas*, which were able to grow on crude oil, were isolated from various hydrocarbon-contaminated sites in Kuwait. They degraded significant amounts of crude oil (> 40 % degradation) (Obuekwe et al., 2009).

Strains A6, A9, A15, K5, K12, M4, Z1, Z4 and Z13 were identified as *Corynebacterium* spp. They were decided to be different depending on their macro- and micro-morphological structures. They degraded used car oil by 60.80 %, 50.00 %, 55.88 %, 41.06 %, 55.45 %, 35.94 %, 47.26 %, 72.97 % and 39.85 %, respectively. On the other hand, they degraded Miri crude oil by 34.42 %, 26.47 %, 34.00 %, 36.00 %, 22.71 %, 23.53 %, 37.07 %, 37.34 % and 54.33 %, respectively. Finally, they degraded Masila crude oil by 11.22 %, 27.67 %, 29.50 %, 18.92 %, 11.22 %, 6.00 %, 25.58 %, 14.49 % and 19.45 % respectively. Oil degrading bacterial cultures isolated from oil contaminated soil samples in a study conducted by Rahman et al. (2002), *Corynebacterium* spp. was selected for this study based on the efficiency of crude oil utilization, and it degraded 43 % of 1 % crude oil concentration.

Strain K3 was identified as *Penicillium* spp.; these fungi degraded used car oil by 51.34 %, Miri crude oil by 37.90 % and Masila crude oil by 23.33 %. *Penicillium zonatum*, grew with crude oil as a sole carbon source (Rudd et al., 1996). In another study *Penicillum* spp. was very effective in degrading crude oil (Cerniglia and Perry, 1973).

Strains M1 and M8 were identified as *Streptomyces* spp. They were differentiated depending on their macro- and micro-morphological structures. They degraded used car oil by 27.72 % and 68.90 % of degradation, respectively, Miri crude oil by 20.03 % and 21.60 %, respectively and Masila crude oil by 7.31 % and 10.18 %, respectively. In a study, a halotolerant *Streptomyces* spp., isolated from field of oil in

Russia, degraded petroleum successfully (Nicholson and Fathepure, 2004). Saadoun (2002) reported the prevalence of members of the genus *Pseudomonas* in the hydrocarbon-polluted soils with the occurrence of other genera, *Enterobacter* and *Acinetobacter*. Radwan et al. (1995) reported a predominance of members of the genus *Pseudomonas* in addition to *Bacillus*, *Streptomyces* and *Rhodococcus*, in the different oil-polluted Kuwaiti Desert soil samples subjected to various types of management.

Strain M9 was identified as *Cladosporium* spp. and it degraded used car oil, Miri and Masila crude oil by 69.42 %, 45.43 % and 21.67 %, respectively. While strain S4 was identified as *Geotrichum* spp. and it degraded used car oil, Miri and Masila crude oil by 53.50 %, 22.54 % and 16.44 %, respectively. Strain M7 was found to be yeast, and it degraded used car oil, Miri and Masila crude oil by 38.86 %, 35.91 % and 4.60 %, respectively. In some reported works, investigated for a period of 18 weeks, the results found that *Cladosporium* spp. and *Geotrichum* spp. were isolated from soil contaminated with crude oil (Obire et al., 2008). *Cladosporium* spp., *Geotrichum* spp, *Penicillium* spp., and two yeast isolates were found to be petroleum-utilizers (Obire and Anyanwu, 2009).

The microbial mixture exerted high effect in hydrocarbon biodegradation, the microbial mixtures were more effective than the single strains in Miri and Masila crude oil. This was supported by many studies; for instance, in a study conducted by Rahman et al. (2002), mixtures were prepared by mixing the oil degrading bacterial cultures isolated from oil contaminated soil samples, *Micrococcus* spp., *Corynebacterium* spp., *Flavobacterium* spp., *Bacillus* spp. and *Pseudomonas* spp. and they were selected for this study based on the efficiency of crude oil utilization. Individual bacterial cultures showed less growth and degradation than did the mixed bacterial mixture. At 1 % crude oil concentration, the mixed bacterial mixture degraded a maximum of 78 % of crude oil. This was followed by 66 % by *Pseudomonas* spp., 59 % by *Bacillus* spp. 49 % by *Micrococcus* spp. 43 % by *Corynebacterium* spp. and 41 % by *Flavobacterium* spp. (Rahman et al., 2002).

4.3 STUDY OF THE EFFECT OF AERATION, ADDED NPK AND MICROBIAL DEGRADERS ON OIL SAMPLES BIODEGRADATION

4.3.1 Percentage of Oil Samples Degradation

i. Used Car Oil

The result of used engine oil biodegradation in different treatment options (aeration, NPK, added microbial degraders and all of them together) after 42 days (Figure 4.7) showed that the group which contained the whole treatment options together had the highest degrading percentage for used car oil (66 %) indicating the biodegradation process, followed by aeration at 61.96 %, and NPK addition (60.91 %). The lowest percentage of degradation was by the added microbial (54.06 %).

As the results shown, the highest percentage of degradation by gathering all treatment options was because of potentiating effect, meaning that the microbes, which were added, were originally isolated from a contaminated area by the same contaminant, which made it already adaptable to use it. In addition, microbial degradation of petroleum hydrocarbon was proven to be a very important factor in the treatment of oil pollution both in aquatic and terrestrial environment (Ibe and Ibe, 1984). The other supporting factor was the aeration which offered the ideal aerobic condition for microorganisms to work and added NPK nutrient was influenced by the growth and proliferation of used car oil utilizing microorganisms in polluted soil as reported by Ubochi et al., (2006). The aeration effect on the biodegradation came in second suggesting that it just offered an aerobic condition for the microbes that were already presented in the soil to work as hydrocarbon degraders. The lowest percentage of degradation was for the container, which contained soil and contaminant with added microbial, and it was even less than the control container which contained soil and contaminant only. This was due to the presence of some microbes in the soil in addition to the added microorganisms in shortage of air and nutrition, which made the microbes compete for the available energy sources, in addition to the accumulation of the toxic metabolites (Zhang et al. 2006). For all these reasons, it was suggested that aeration and nutrient addition are vital for microbial growth and activity in the hydrocarbon contaminated soils.

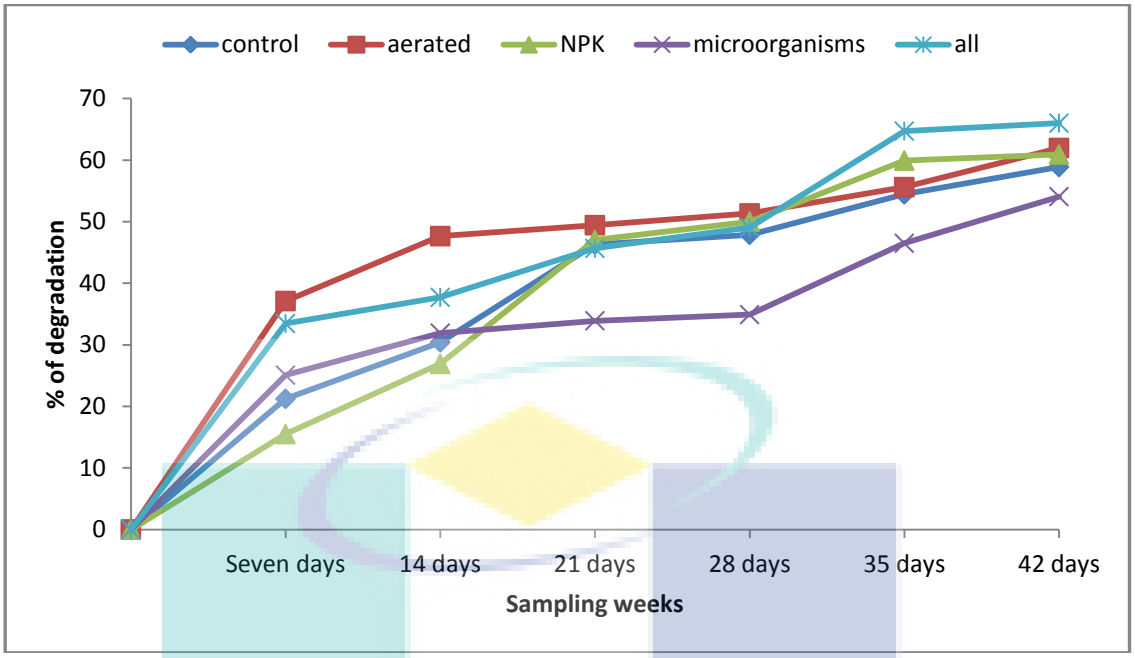


Figure 4.7 Percentage of degradation of used car oil by the effect of aeration added NPK and added microbial degraders after 42 days. (All) refer to all the treatments together in the same container

ii. (Masila) Crude Oil

The study of the effect of aeration, added NPK, added isolated microbial degraders and all of them together on Masila crude oil (Figure 4.8) showed that the group which was aerated continuously resulted in the highest degrading percentage for Masila crude oil (56.62 %), followed by NPK added container with a similar percentage of degradation (54.93 %), then the microbial mixture added container with 53.69 % of degradation. The lowest percentage of degradation was by the control container (46.25 %).

From the above results, it can be suggested that the highest percentage of degradation by the aeration was because the ventilation, which offered the ideal aerobic condition necessary for microbial growth, in addition to the enhancement of the crude oil evaporation process due to the soil tilling, which affected this type of light crude oil that contained easily evaporated compounds. This was followed by added NPK nutrient which influenced the growth and proliferation of crude oil utilizing microorganisms in polluted soil as it was reported by Ubochi et al. (2006). In contrast, the least percentage

of degradation was for the control container due to lack of necessary nutrients and oxygen for the microbial survival, which affected the biodegradation process by affecting the microorganisms the present indigenously in the soil.

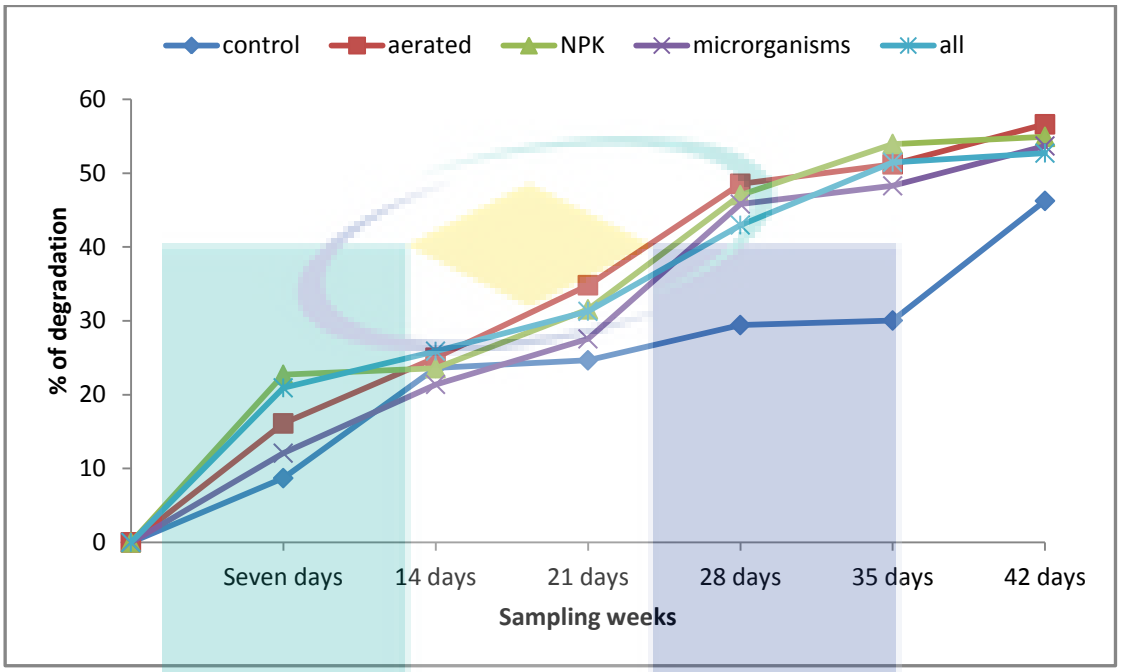


Figure 4.8 Percentage of degradation of Masila crude oil by the effect of aeration added NPK and added microbial degraders after 42 days. (All) refer to all the treatments together in the same container.

4.3.2 Total microbial heterotrophic count (THC).

i. Used Car Oil

The total heterotrophic count (THC) of microbial population is presented in Figure 4.9 in different treatment option of used car oil (aeration, added NPK, added microbial and all of them together) in 42 days on nutrient agar media was studied. The THC of all containers at zero time were close to each other (9.9×10^3, 1.1×10^4, 1.2×10^4, 9.8×10^3 and 1.3×10^4 cfu/g) for the control container, aerated, NPK, added microbes and all factors of added containers, respectively. This was because all the soil samples were collected from the same area.

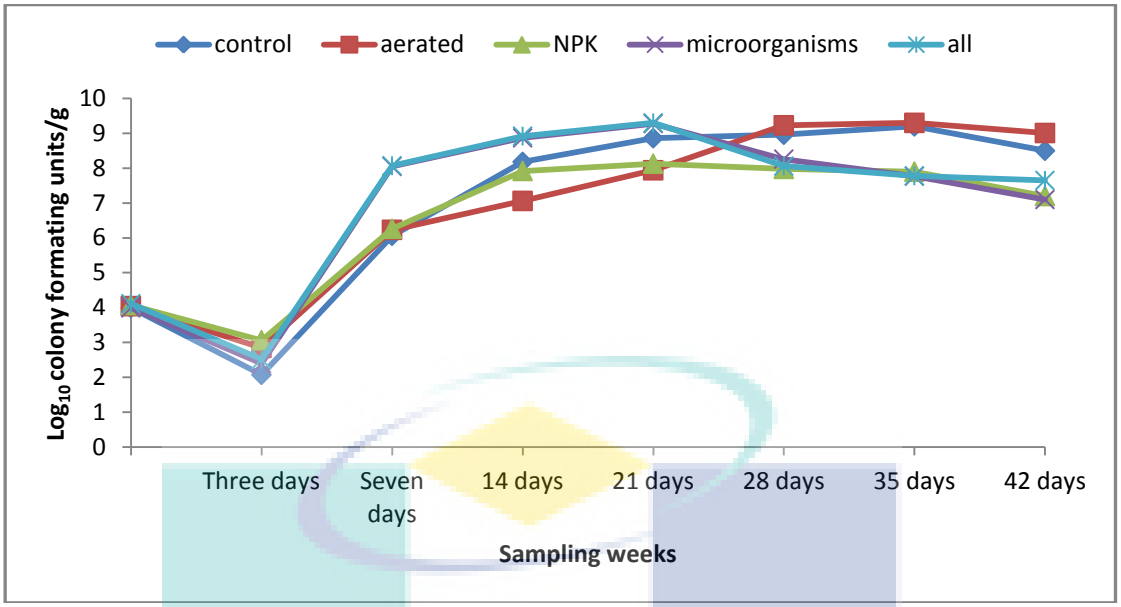


Figure 4.9 Total heterotrophic count of microbial population in different treatment option of used car oil (aeration, NPK and added microbial degraders after 42 days. (All) refer to all the treatments together in the same container.

After three days of treating the added used car oil, the total count (THC) was measured again, the results showed a decrease in THC in all treated containers as follow: 1.2 x 10^2, 7.0 x 10^2, 1.1 x 10^3, 2.5 x 10^2 and 3.3 x 10^2 cfu/g for control, aerated, NPK, added microbes and all factors of the added containers, respectively as a response to used car oil contamination, and this sequence followed for all treatment options. The THC increased again from the day seven to the day 21 for the soil treated by all treatment options, microbial treated and NPK treated containers, while it was increased to the day 35 for aerated and control containers before reducing again in the day 42. At the end, the cumulative THC were 1.6 x 10^9, 2.0 x 10^9, 8.0 x 10^7, 6 x 10^7, 6.1 x 10^7 cfu/g for control, aerated, NPK, added microbes and all factors of the added containers, respectively.

The decrease in the count of total heterotrophic microorganisms in the contaminated soil in the first three days can be due to inhibition of some microbes. This is because of the toxic effect of some used car oil components (Zhang et al., 2006) and oxygen deficiency as well as the changes in the balance of carbon/inorganic nutrient for original microbial population in the soil (Atlas, 1984), that was caused by the presence

of used car oil. While the THC increased to the day 21 for the control container which contained only soil and oil samples, NPK treated container, microbial treated container as well as the container with all treatment options caused by the adaption process of the microbes which encouraged it to start using used car oil before it was decreased again due to the accumulation of metabolites and nutrient deficiency. In the aerated container, the THC increase continued to the day 35 which suggested the importance of the ventilation process to biodegrade the hydrocarbon sources using the aerobic biodegradation in the absence of the competition by other added microorganisms rather that the indigenous presence in the soil.

ii. (Masila) Crude Oil

In Figure 4.10, the total heterotrophic count of microbial population was presented in different treatment options of crude oil in 42 days on nutrient agar media. The total heterotrophic count (THC) of all containers at zero time were 8.9×10^3, 1.0×10^4, 9.0×10^3, 8.7×10^3 and 9.6×10^3 cfu/g for control, aerated, NPK, added microbes and all factors of the added containers, respectively. After three days of adding crude oil, the total count (THC) was recorded again, the results showed a decrease in THC in all treated containers as follow: 5.2×10^2, 4.8×10^2, 3.5×10^2, 4.5×10^2 and 8.7×10^2cfu/g for control, aerated, NPK, added microbes and all treatment options containers, respectively. This was as a response to toxic effect of crude oil contamination, and this sequence was followed for all treatment options. The THC increased again from the day seven to the day 21 before it decreased again in the day 42. At the end the cumulative THC were 3.0×10^7, 5.4×10^7, 6.9×10^6, 3.9×10^7, 3.2×10^6 cfu/g for control, aerated, NPK, added microbes and all factors of the added containers, respectively.

The drop in the total heterotrophic count in the contaminated soil in the first three days can be attributed to selective inhibition of members of the microbial community. This was caused by the crude oil toxicity and also as a result of reduced aeration and nutrient shortage for indigenous population. The highest THC was for the aerated container due to the ideal aerobic condition for microbial growth, while the lowest THC for container with all treatment options due to the microbial competition and toxin accumulation.

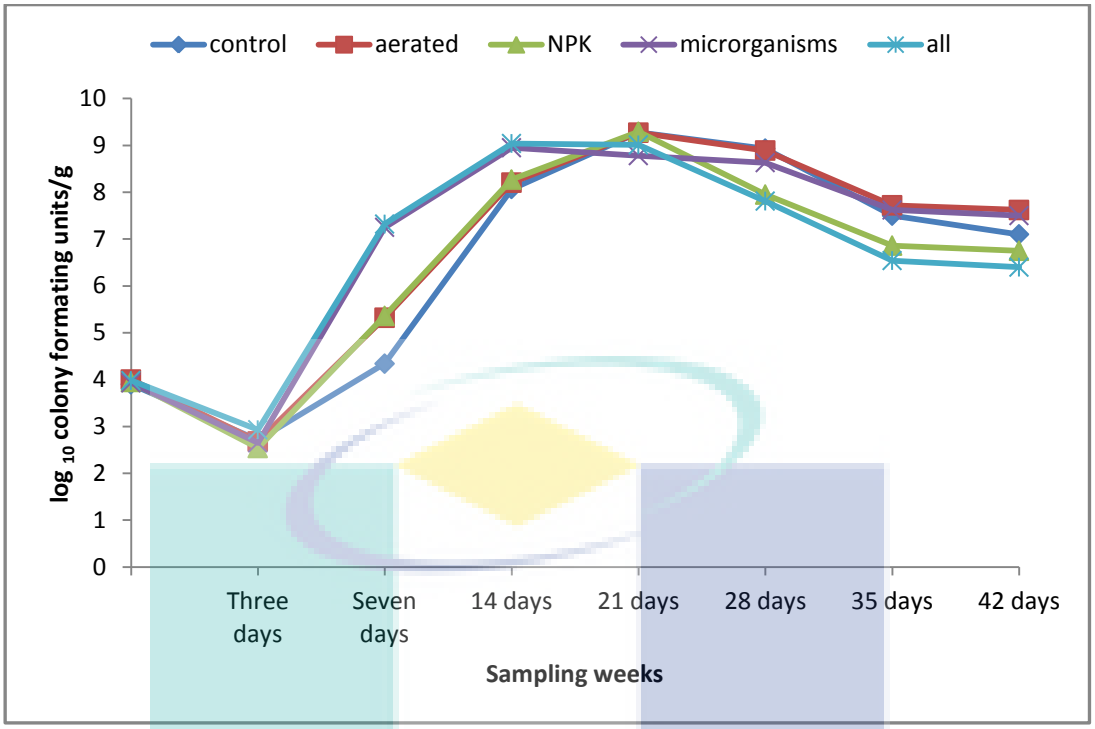


Figure 4.10 Total heterotrophic count of microbial population in different treatment option of Masila crude oil (aeration, NPK and added microbial degraders after 42 days. (All) refer to all the treatments together in the same container.

4.3.3 Hydrocarbon utilizing microbial count (HUM)

i. Used Car Oil

Figure 4.11 shows the hydrocarbon utilizing microbial count (HUM) result. The HUM count increased over the period of the study. At three days, the HUM of microbial added container and all option treated container were higher than the other treatment options (1.0 x 10^4 and 1.2 x 10^4 cfu/g), respectively, because these two containers contained added microbial strains and isolated from contaminated areas which made it adaptable to hydrocarbon biodegradation. At the end of the study (after 42 days), the HUM count were 2.0 x 10^7, 2.0 x 10^7, 2.0 x 10^6, 2.0 x 10^6 and 2.8 x 10^7 cfu/g for control, aerated, added NPK, added microbial and all option treatment containers, respectively. This shows that the soil treated with all treatment options gave the highest number of hydrocarbon utilizing microorganisms. The increase in HUM count in response to the adding of microbial mixture, continuous aeration and supplementing

with NPK was a good factor, which affected the microbial growth by offering the high number of microbes which were already adapted to degrade the used car oil, offering the necessary aerobic condition for microbial growth, in addition to the nutrient necessary to the microbes.

The lowest HUM count was for containers supplemented with microbes and NPK due to the oxygen deficiency and microbial competition which made the microbial growth limited at the end of the study.

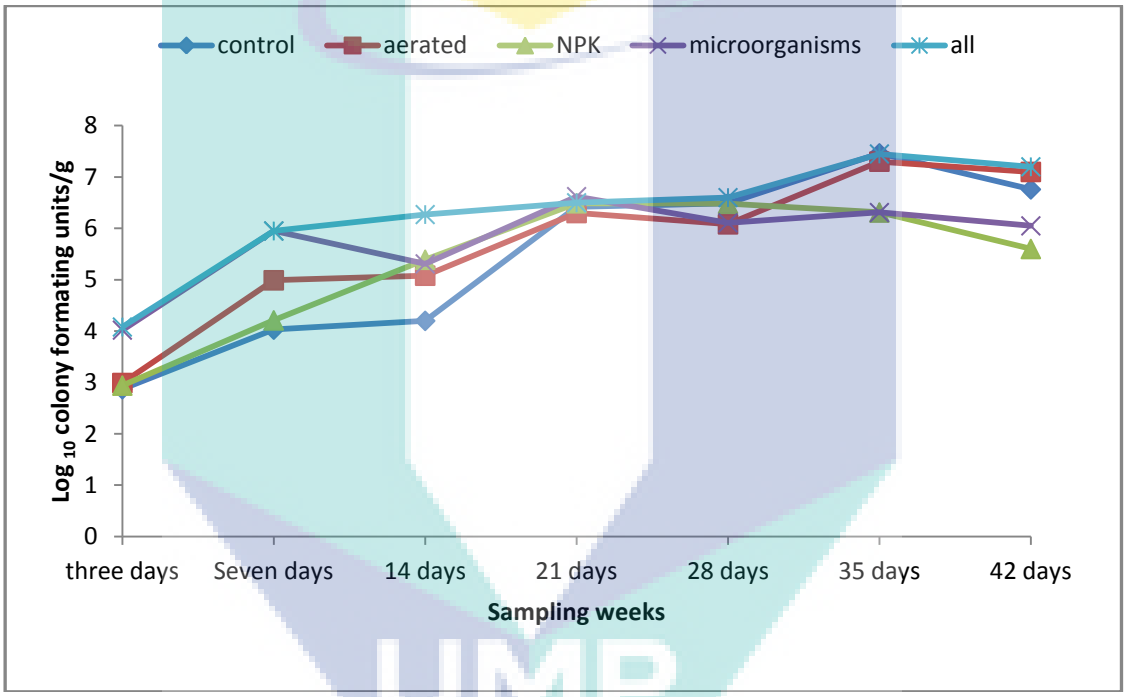


Figure 4.11 Total count of hydrocarbon utilizing microorganisms in different treatment option of used car oil (aeration, added NPK, added microbial degraders and all of them together) after 42 days. (All) refer to all the treatments together in the same container.

ii. (Masila) Crude Oil

Hydrocarbon utilizing microbial count (HUM) result is shown in Figure 4.12. In general, the HUM count increased during the study period. At three days, the HUM of the container with added microbial as well as the container with all treatment options were higher than the other containers (1.0×10^3 and 1.8×10^4 cfu/g), respectively, because these two containers contained hydrocarbon use adapted microbial strains

which were isolated from contaminated areas. At the end of the study (after 42 days), the HUM count were 8.7 x 10^3, 1.8 x 10^6, 3.5 x 10^4, 2.3 x 10^5 and 6.6 x 10^4 cfu/g for control, aerated, added NPK, added microbial and all option treatment containers, respectively. This shows that the aerated container gave the highest number of hydrocarbon utilizing microorganisms. The increase in HUM count was in response to the aerobic conditions necessary for microbial growth. The lowest HUM count was for the control containers due to the oxygen and nutrient deficiency which made the microbial growth limited at the end of the study.

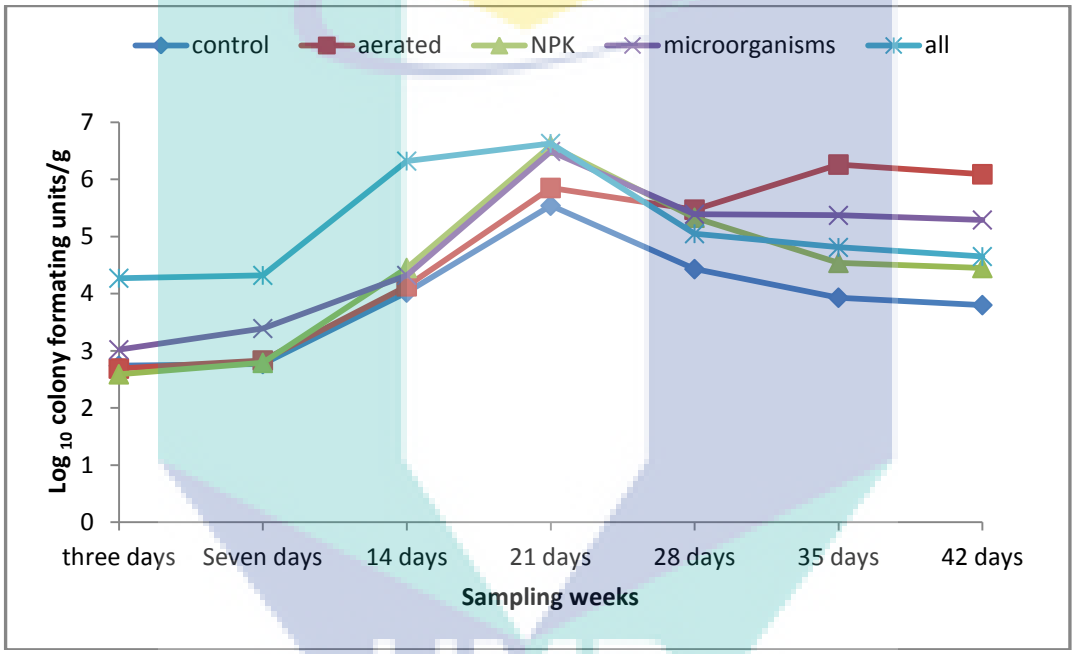


Figure 4.12 Total counts of hydrocarbon utilizing microorganisms in different treatment option of Masila crude oil (aeration, added NPK, added microbial degraders and all of them together) after 42 days. (All) refer to all the treatments together in the same container.

It was found from the results, that total microbial count at different times were directly proportional with the percentage of degradation. In used car oil experiment, the total hydrocarbon utilizing microbes reached the highest result at the day 35, at the same time, the percentage of used car oil degradation was at the highest rate in the same period of time. Similarly, in Masila crude oil experiment, the total count of hydrocarbon utilizing microbes increased to the end of the study, the percentage of Masila crude oil degradation increased at the same period of time. This can be attributed to the

responsibility of the microorganisms for the degradation. Where, the high microbial count which was concomitant with the high percentage of degradation was a clear indication to the microbial effect. This was supported by Ekpo and Udofia (2008) findings. Where, the high microbial growth pattern in crude oil contaminated soil was attributed to the high percentage of biodegradation.

Several factors are affecting hydrocarbon biodegradation process such as concentration and type of contaminant (Boethling and Alexander, 1979), application of nutrients such as nitrogen and phosphorous was shown to be effective in accelerating the biodegradation process (Pritchard and Costa, 1991) and repeated aeration of the polluted soil may further increase the biodegradation of hydrocarbon (Syafruddin et al., 2010).

The n-alkenes based on their chemical nearness combination are divided into six classes: < C_{13}, C_{13}-C_{16}, C_{17}-C_{21}, C_{22}-C_{25}, C_{26}-C_{29}, C_{29}-C_{36}. The first class includes normal alkanes which are smaller than C13. As these hydrocarbons evaporate in normal conditions, so they cannot be measured (Chorom et al., 2010). This was the reason that the control group in this study lost some of hydrocarbon contents compared with the percentage in zero time. The effect of time on used car oil and petroleum degradation was significant during 42 days of the study. The used car oil and petroleum degradation rate decreased with increasing time, and this observation corresponded with the microorganisms growing results. The highest microbial growing was determined after 35 days, at the same time the percentage of degradation of used car oil and Masila crude oil were 64.71 % and 51.21 %, respectively. At the end of the study, the percentage of degradation was 66 % and 56.62 %, respectively; because of the presence of fast decomposed normal paraffin, organic nutrition materials and microbial activity, and as a result, hydrocarbons degradation was at a maximum rate.

Sang-Hawn et al. (2007) made a similar observation and concluded that hydrocarbon degrading bacterial populations increased rapidly during the first four weeks of 14 weeks testing period. They proposed this finding that it may be considered as an indicator for the feasibility of oil-contaminated soils bioremediation. However, with increasing of time, due to the oil-resistant components with high chain and within

less remaining nutrients, the bacteria growth and oil degradation decreased (Schaefer and Juliane, 2007). Ramsay et al. (2000) in their study of bioremediation on microbial population in oil sediments observed that continual ventilation and fertilizer increment had considerable effect on the growth of hydrocarbonic degrading bacteria in soil. Van Gestel et al. (2001) reported a significant increment of the oil-polluted soil bioremediation in bacteria population. Authors in study reported that with increasing fertilizers and soil ventilation in addition to microbial addition during bioremediation and at the time of increasing heterotrophy grows, petroleum degradation has also increased and soil pollution showed a decrease between 45-60 % (Rahman et al., 2002).

4.3.4 Soil pH Changes for Used Car Oil

The pH of soil in all containers was measured at zero time, after three days and then every seven days for the period of the study. The results of soil pH during the period of sampling are presented in Table 4.4. The pH was acidic between 5.45 and 4.94 for all samples during the study period. There was a slight decrease in pH of all treatment options, indicating the biodegradation process, which mainly resulted in acidic products. The container with NPK had the least pH value due to the effect of the addition of Nitrogen, phosphorous and potassium on pH in general after dissolving of these elements in water within the study time.

Table 4.3: Used car oil pH change during the period of study

Code	Zero time	3 days	7 days	14 days	21 days	28 days	35 days	42 days
Use car oil control	5.45	5.28	5.20	5.17	5.13	5.09	5.08	5.06
Use car oil (aeration)	5.39	5.31	5.13	5.10	5.08	5.04	5.04	5.03
Use car oil NPK	5.40	5.25	5.12	5.10	5.08	4.99	4.94	**4.94**
Use car oil microorganism	5.42	5.36	5.23	5.20	5.17	5.15	5.11	5.09
Use car oil all	5.33	5.25	5.22	5.18	5.14	5.05	5.01	5.00

4.3.5 Soil pH changes for Masila crude oil

The pH of soil in all containers was measured in the same above mentioned way. Table 4.5 shows the results of soil pH during the period of sampling. It was obvious that the soil pH was acidic between 5.50 and 4.70 for all samples. There was a decrease in pH of all treatment options, which pointed to the biodegradation process, that result mainly in acidic products. The NPK added container had the least pH value (4.70) due to nitrogen, phosphorous and potassium effect on pH.

Table 4.4: Masila Crude oil pH change during the period of study

Code	Zero time	3 days	7 days	14 days	21 days	28 days	35 days	42 days
Crude oil control	5.45	5.40	5.35	5.2 4	5.17	5.06	5.06	5.05
Crude oil (aeration)	5.50	5.30	5.10	5.01	4.86	4.80	4.79	4.77
Crude oil NPK	5.39	5.21	5.11	5.04	4.90	4.83	4.72	**4.70**
Crude oil microorganisms	5.42	5.32	5.28	5.27	5.17	5.14	5.09	5.08
Crude oil all	5.33	5.25	5.24	5.19	5.02	4.89	4.76	4.75

In general, during this study period, the pH decreased (Table 4.4 and 4.5), this might be because of the production of acidic metabolites due to crude oil biodegradation product, which are mainly acids and methane (Ian et al., 2003). In a study conducted by Obuekwe and Al-Zarban (1998), it was found that during the degradation of crude oil by the isolated microbial mixture, the pH declined continuously during incubation, suggesting the production of acidic products. Similar observations were made and reported by Ayotamuno et al. (2006); Tisdale and Nelson (1975), where the decrease in pH was suggested that it might have resulted from production of acid radicals.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

The ability of various indigenous microorganisms, especially those isolated from polluted sites, to metabolize hydrocarbons is well known. In this study, microorganisms that are able to grow in the presence of used car oil were isolated from the contaminated soil sites in Kuala Lumpur area, Malaysia. Selective mediums (agars containing crude oil, used car oil and benzene) were used to select oil samples degraders. Growth of the isolated microorganisms on used car oil and crude oil containing media indicated the presence of hydrocarbon and oil-degrading activities in isolated microbial strains. Each of the research goals, addressed in chapter 1 was individually evaluated and successfully executed. The following conclusions have been reached: of the 22 microorganisms isolated from soil of different car workshops areas selected for studying hydrocarbon biodegradation, the two bacterial species found, *Corynebacterium* spp., and *Streptomyces* spp., in addition to one fungus, *Cladosporium* spp., were the highest affecting degraders for used car oil. On the other hand, two bacterial species, *Corynebacterium* spp., and *Pseudomonas* spp., as well as one fungus, *Cladosporium* spp., were found to be the highest affecting degraders for Miri crude oil. Finally, in Masila crude oil, the bacterial species, *Klebsiella pneumonia*, *Corynebacterium* spp., and *Pseudomonas* spp. were the highest degraders. The highest degradability was detected on used car oil, which was suggested to be due to the adaption of microorganisms to this hydrocarbon where it was the main contaminant in the car workshop that the microbes were originally isolated from.

Generally, in hydrocarbon degradation tests using microbial mixtures, it was found that the microbial mixtures that were prepared by mixing the microorganisms isolated from

the same car workshop were the highest degraders. In Miri crude oil biodegradation, the highest effect was for MS microbial mixture. At the same time, Masila crude oil percentage of degradation was enhanced when microbial mixtures were used compared to the use of single strains, the highest affecting mixture compared to the other mixtures was Smix. This indicates that the microbial mixtures were a more successful choice to be used in crude oil biodegradation. In used car oil biodegradation test, the highest affecting mixture was Zmix. It can be concluded that the microbial mixture MS for Miri crude oil, Smix for Masila crude oil, and Zmix for used car oil are good candidates for application in the field to study the biodegradation effect.

In the study of the effect of aeration, added NPK and added microbial degraders on biodegradation process, continuous ventilation played essential role in Masila crude oil biodegradation, In other words, all gathered factors were effective in used car oil biodegradation, which suggests field treatment by aeration, nutrition and added microbial degraders mixture to achieve fast clean up results with the lowest costs.

5.2 RECOMMENDATIONS

Depending on the result of this study, we recommend the following:

1- Choosing the best microbial mixture and formulate it in a product in order to apply it to larger study.

2- For future studies, the microorganisms can be identified in a molecular level and determine the gene responsible on the biodegradation process.

3- In a rich natural microbial environment such as Malaysia, we recommend following the economic options and just promote the microbial degradation for hydrocarbon contaminants such as continuous ventilation and nutrient addition.

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APPENDIX A

GRAM STAINING PROCEDURE

1. Place a loop of 0.85% normal saline onto a clean slide.
2. Smear a colony of selected bacteria onto the drop of saline on the slide.
3. Fixed the smear by heating and air dried before staining.
4. Stain the smear with few drops of Crystal Violet for 1 minute.
5. Wash with running water.
6. Flood the smear with Lugol's Iodine for 1 minute.
7. Wash the smear with a few drops of acetone for 2-3 seconds
8. Rinse with running water.
9. Stained the smear with diluted Carbol Fuchin solution for 1 minute.
10. Wash with running tap water.
11. Blot-dry the smear by a piece of filter paper and leave it for 5 minutes to dry then examine it under the microscope.

APPENDIX B

BBL CRYSTAL™ IDENTIFICATION SYSTEM (GRAM-NEGATIVE-ID KIT)

BBL Crystal™ Identification Systems (Gram-negative -ID Kit) (BD) (Becton, Dickinson and company).

A pure culture of each isolates was cultured on TSA and incubated at 35°C for 18 hours. Aseptically, 2-5 colonies were collected by and added to inoculation fluid tube and adjusted to Mcfarland No. 0.5 turbidity standard. The inoculation fluid was then poured into target area of the BBL Crystal base and rolled until all of the wells were filled and then covered with lid cover tightly. Then the inoculated panels were incubated at 35°C for 24 hours. All the panels were read with BBL Crystal panel viewer, and the results were entered into BBL Crystal software to obtain the bacterial species identification.

APPENDIX C

ISOLATED MICROBIAL MIXTURES CONTENT ACCORDING TO THE SITE OF ISOLATION (CAR WORKSHOPS)

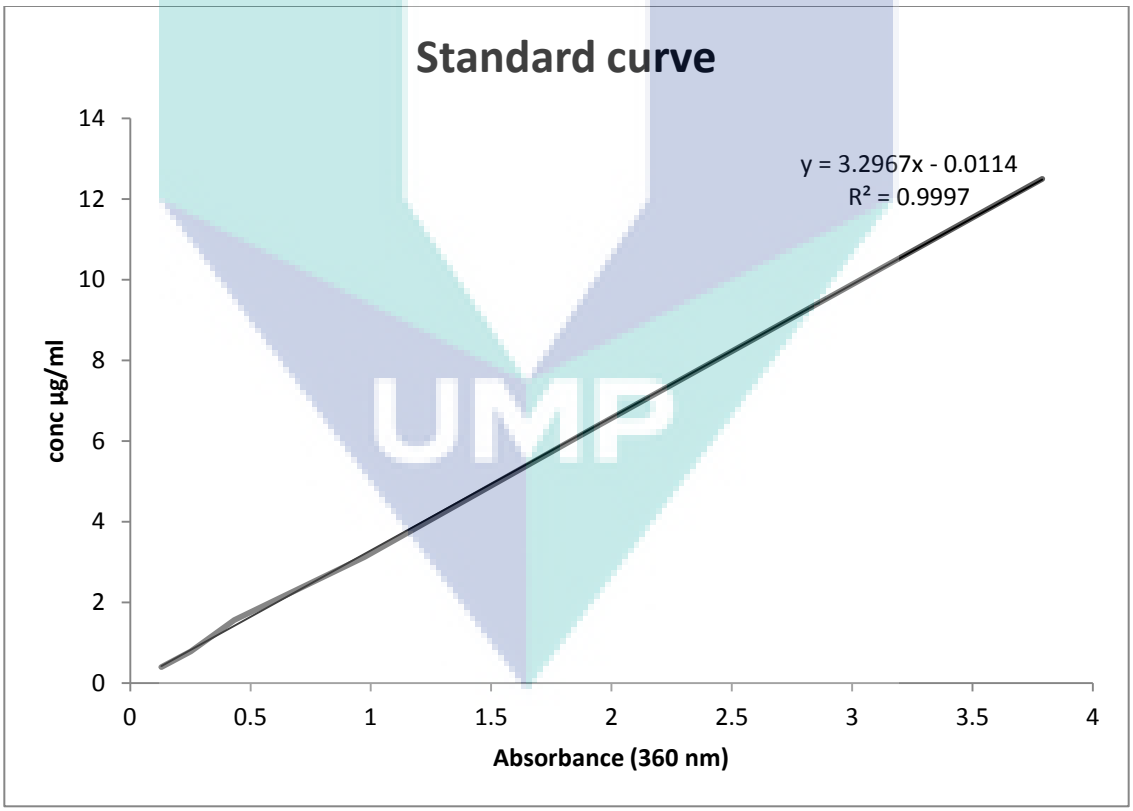
Area	Group	Content
Selangor	A mix	*Achrombacter* A1 , *Aeromonas* A2, *Acinetobacter* A3, *Pseudomonas*, A5, *Corynebacterium* A6, *Corynebacterium* A9 ,*Corynebacterium* A15
Kuala Lumpur	K mix	*Pseudomonas* K1, *Penicillium* K3, *Corynebacterium* K5 *Enterobacteriaceae* K11 , *Corynebacterium* K12
Rawang	M mix	*Streptomyce*, M1, *Corynebacterium* M4, yeast cells M7, *Streptomyces* M8, *Cladosporum* M9
Serdang	S mix	*Actinobacillus* S1, *Geotrichum* S4
Kajang	Z mix	*Corynebacterium* Z1, *Corynebacterium* Z4, *Corynebacterium* Z13
Rawang, Serdang	MS	*Streptomyces* M1, *Corynebacterium* M4, yeast cells M7, *Streptomyces* M8, *Cladosporum* M9, *Actinobacillus* S1, *Geotrichum* S4
Kajang, Serdang	ZS	*Corynebacterium* Z1, *Corynebacterium* Z4, *Corynebacterium* Z13, *Actinobacillus* S1, *Geotrichum* S4
Kuala Lumpur, Selangor	KA	*Pseudomonas* K1, *Penicillium* K3, *Corynebacterium* K5, *Enterobacteriaceae* K11, *Corynebacterium* K12, *Achrombacter* A1, *Aeromonas* A2, *Acinetobacter* A3, *Pseudomonas* A5, *Corynebacterium* A6, *Corynebacterium* A9, *Corynebacterium* A15
Selangor, Rawang	AM	*Achrombacter* A1, *Aeromonas* A2, *Acinetobacter* A3, *Pseudomonas* A5, *Corynebacterium* A6, *Corynebacterium* A9, *Corynebacterium* A15, *Streptomyces* M1, *Corynebacterium* M4, yeast cells M7, *Streptomyces* M8, *Cladosporum* M9
Selangor, Kajang	AZ	*Achrombacter* A1, *Aeromonas* A2, *Acinetobacter* A3, *Pseudomonas* A5, *Corynebacterium* A6, *Corynebacterium* A9, *Corynebacterium* A15, *Corynebacterium* Z1,

		Corynebacterium Z4, *Corynebacterium* Z13
Selangor, Serdang	AS	*Achrombacter* A1, *Aeromonas* A2, *Acinetobacter* A3, *Pseudomonas* A5, *Corynebacterium* A6, *Corynebacterium* A9, *Corynebacterium* A15, *Actinobacillus* S1, *Geotrichum* S4
Kajang, Kuala Lumpur	ZK	*Corynebacterium* Z1, *Corynebacterium* Z4, *Corynebacterium* Z13, *Pseudomonas* K1, *Penicillium* K3, *Corynebacterium* K5, *Enterobacteriaceae* K11, *Corynebacterium* K12
Rawang, Kuala Lumpur	MK	*Streptomyces* M1, *Corynebacterium* M4, yeast cells M7, *Streptomyces* M8, *Cladosporum* M9, *Pseudomonas* K1, *Penicillium* K3, *Corynebacterium* K5, *Enterobacteriaceae* K11, *Corynebacterium* K12
Rawang, Kajang	MZ	*Streptomyces* M1, *Corynebacterium* M4, yeast cells M7, *Streptomyces* M8, *Cladosporum* M9, *Corynebacterium* Z1, *Corynebacterium* Z4, *Corynebacterium* Z13
Serdang, Kuala Lumpur	SK	*Actinobacillus* S1, *Geotrichum* S4, *Pseudomonas* K1, *Penicillium* K3, *Corynebacterium* K5, *Enterobacteriaceae* K11, *Corynebacterium* K12
Selangor, Kuala Lumpur	AK	*Achrombacter* A1, *Aeromonas* A2, *Acinetobacter* A3, *Pseudomonas* A5, *Corynebacterium* A6, *Corynebacterium* A9, *Corynebacterium* A15, *Pseudomonas* K1, *Penicillium* K3, *Corynebacterium* K5, *Enterobacteriaceae* K11, *Corynebacterium* K12

APPENDIX D

MIRI CRUDE OIL STANDARD CURVE

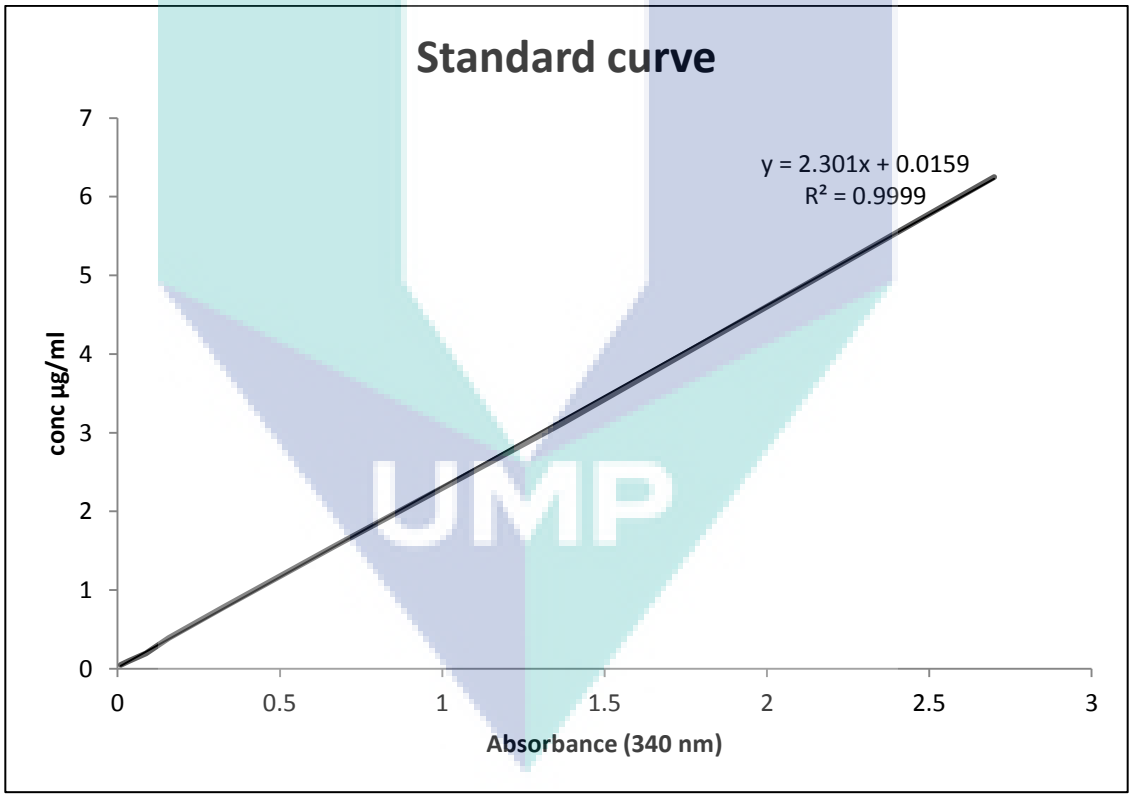
abs	3.79	1.905	0.974	0.433	0.252	0.129
conc µg/ml	12.5	6.25	3.12	1.56	0.781	0.39



APPENDIX E

USED CAR OIL STANDARD CURVE

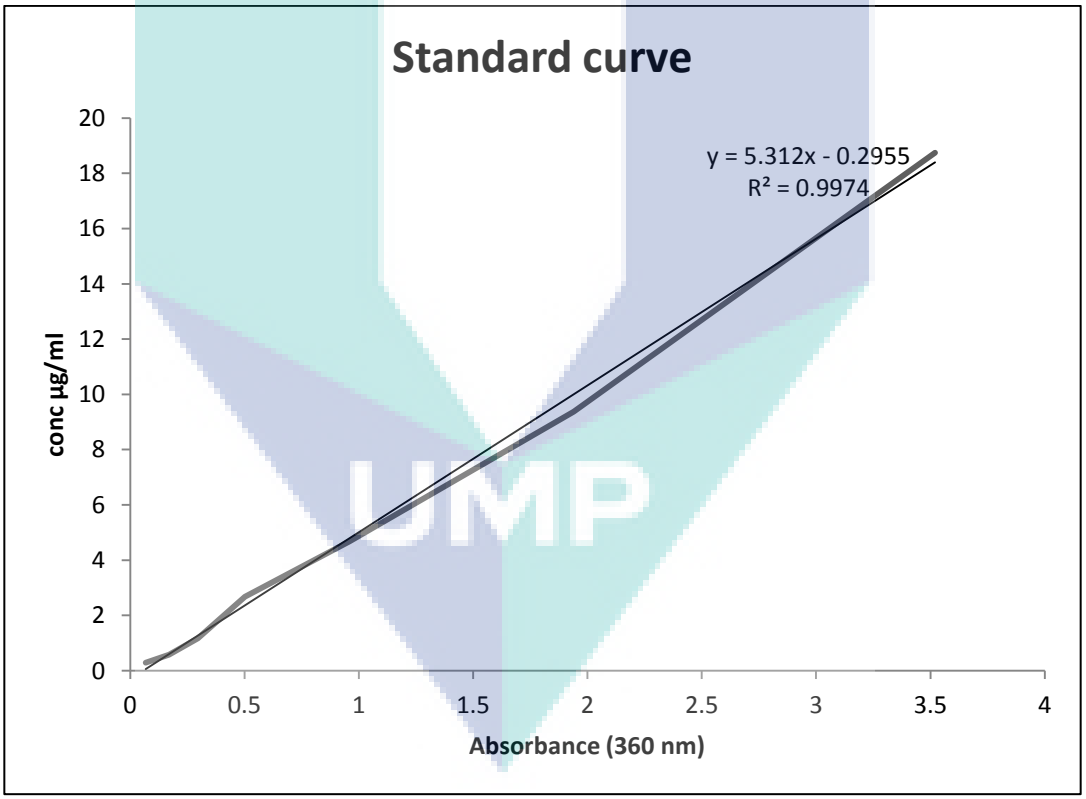
abs	2.7	1.372	0.67	0.326	0.157	0.087	0.034	0.009
conc µg/ml	6.25	3.125	1.562	0.781	0.39	0.195	0.0976	0.0488



APPENDIX F

MASILLA CRUDE OIL STANDARD CURVE

abs	3.52	1.94	0.96	0.501	0.296	0.17	0.067
conc µg/ml	18.75	9.37	4.68	2.68	1.17	0.585	0.292



APPENDIX G

STATISTICAL ANALYSIS

Multiple Comparisons

Microbial single strains used car oil

Dunnett t (2-sided)[a]

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	24	42.86000*	.51948	.000	41.2656	44.4544
2	24	35.33667*	.51948	.000	33.7422	36.9311
3	24	61.80667*	.51948	.000	60.2122	63.4011
4	24	42.41667*	.51948	.000	40.8222	44.0111
5	24	54.94667*	.51948	.000	53.3522	56.5411
6	24	45.04000*	.51948	.000	43.4456	46.6344
7	24	50.49000*	.51948	.000	48.8956	52.0844
8	24	42.13000*	.51948	.000	40.5356	43.7244
9	24	45.78333*	.51948	.000	44.1889	47.3778
10	24	35.53333*	.51948	.000	33.9389	37.1278
11	24	27.51667*	.51948	.000	25.9222	29.1111
12	24	50.15000*	.51948	.000	48.5556	51.7444
13	24	22.30667*	.51948	.000	20.7122	23.9011
14	24	30.67333*	.51948	.000	29.0789	32.2678
15	24	33.29333*	.51948	.000	31.6989	34.8878
16	24	63.33333*	.51948	.000	61.7389	64.9278
17	24	64.31333*	.51948	.000	62.7189	65.9078
18	24	44.18000*	.51948	.000	42.5856	45.7744
19	24	47.92667*	.51948	.000	46.3322	49.5211
20	24	41.75333*	.51948	.000	40.1589	43.3478
21	24	66.69000*	.51948	.000	65.0956	68.2844
22	24	34.30000*	.51948	.000	32.7056	35.8944
23	24	52.99667*	.51948	.000	51.4022	54.5911

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

*. The mean difference is significant at the 0.05 level.

I group for the microbial single strains, J group for control.

Multiple Comparisons

Microbial mixtures used car oil

Dunnett t (2-sided)[a]

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	16	55.62000*	.00354	.000	55.6094	55.6306
2	16	51.47000*	.00354	.000	51.4594	51.4806
3	16	44.91000*	.00354	.000	44.8994	44.9206
4	16	54.46000*	.00354	.000	54.4494	54.4706
5	16	44.16000*	.00354	.000	44.1494	44.1706
6	16	27.28000*	.00354	.000	27.2694	27.2906
7	16	36.13000*	.00354	.000	36.1194	36.1406
8	16	49.78000*	.00354	.000	49.7694	49.7906
9	16	52.64000*	.00354	.000	52.6294	52.6506
10	16	29.30000*	.00354	.000	29.2894	29.3106
11	16	37.82000*	.00354	.000	37.8094	37.8306
12	16	43.60000*	.00354	.000	43.5894	43.6106
13	16	51.57000*	.00354	.000	51.5594	51.5806
14	16	65.32000*	.00354	.000	65.3094	65.3306
15	16	66.49000*	.00354	.000	66.4794	66.5006

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

*. The mean difference is significant at the 0.05 level.

I group for the microbial mixtures, J group for control.

Multiple Comparisons

Microbial single strains Miri crude oil

Dunnett t (2-sided)[a]

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	24	27.72667*	1.71427	.000	22.4651	32.9883
2	24	2.42000	1.71427	.859	-2.8416	7.6816
3	24	36.85667*	1.71427	.000	31.5951	42.1183
4	24	43.11667*	1.71427	.000	37.8551	48.3783
5	24	29.25000*	1.71427	.000	23.9884	34.5116
6	24	21.35333*	1.71427	.000	16.0917	26.6149
7	24	30.14667*	1.71427	.000	24.8851	35.4083
8	24	10.62000*	1.71427	.000	5.3584	15.8816
9	24	33.28000*	1.71427	.000	28.0184	38.5416
10	24	31.04000*	1.71427	.000	25.7784	36.3016
11	24	27.22000*	1.71427	.000	21.9584	32.4816
12	24	18.00333*	1.71427	.000	12.7417	23.2649
13	24	15.32000*	1.71427	.000	10.0584	20.5816
14	24	18.18667*	1.71427	.000	12.9251	23.4483
15	24	30.85000*	1.71427	.000	25.5884	36.1116
16	24	16.54667*	1.71427	.000	11.2851	21.8083
17	24	40.28667*	1.71427	.000	35.0251	45.5483
18	24	22.00333*	1.71427	.000	16.7417	27.2649
19	24	17.41667*	1.71427	.000	12.1551	22.6783
20	24	32.21333*	1.71427	.000	26.9517	37.4749
21	24	32.21333*	1.71427	.000	26.9517	37.4749
22	24	49.31000*	1.71427	.000	44.0484	54.5716
23	24	52.39333*	1.71427	.000	47.1317	57.6549

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

*. The mean difference is significant at the 0.05 level.

I group for the microbial single strains, J group for control.

Multiple Comparisons

Microbial mixture Miri crude oil

Dunnett t (2-sided)[a]

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	16	71.89667*	.12829	.000	71.5110	72.2824
2	16	68.71000*	.12829	.000	68.3243	69.0957
3	16	65.42000*	.12829	.000	65.0343	65.8057
4	16	75.44000*	.12829	.000	75.0543	75.8257
5	16	51.95000*	.12829	.000	51.5643	52.3357
6	16	72.60000*	.12829	.000	72.2143	72.9857
7	16	76.60333*	.12829	.000	76.2176	76.9890
8	16	69.44000*	.12829	.000	69.0543	69.8257
9	16	68.74000*	.12829	.000	68.3543	69.1257
10	16	68.10000*	.12829	.000	67.7143	68.4857
11	16	35.31000*	.12829	.000	34.9243	35.6957
12	16	60.70000*	.12829	.000	60.3143	61.0857
13	16	65.44000*	.12829	.000	65.0543	65.8257
14	16	74.67333*	.12829	.000	74.2876	75.0590
15	16	43.29000*	.12829	.000	42.9043	43.6757

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

*. The mean difference is significant at the 0.05 level.

I group for the microbial mixtures, J group for control.

Multiple Comparisons

Microbial single strains Masila crude oil

Dunnett t (2-sided)[a]

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	24	12.71000*	.01167	.000	12.6742	12.7458
2	24	18.93000*	.01167	.000	18.8942	18.9658
3	24	32.11000*	.01167	.000	32.0742	32.1458
4	24	26.50000*	.01167	.000	26.4642	26.5358
5	24	9.51333*	.01167	.000	9.4775	9.5491
6	24	25.97667*	.01167	.000	25.9409	26.0125
7	24	27.81000*	.01167	.000	27.7742	27.8458
8	24	4.01333*	.01167	.000	3.9775	4.0491
9	24	21.64000*	.01167	.000	21.6042	21.6758
10	24	17.23000*	.01167	.000	17.1942	17.2658
11	24	12.02000*	.01167	.000	11.9842	12.0558
12	24	9.51333*	.01167	.000	9.4775	9.5491
13	24	5.62667*	.01167	.000	5.5909	5.6625
14	24	4.31000*	.01167	.000	4.2742	4.3458
15	24	2.91000*	.01167	.000	2.8742	2.9458
16	24	8.48000*	.01167	.000	8.4442	8.5158
17	24	19.97000*	.01167	.000	19.9342	20.0058
18	24	3.01000*	.01167	.000	2.9742	3.0458
19	24	14.75333*	.01167	.000	14.7175	14.7891
20	24	23.89000*	.01167	.000	23.8542	23.9258
21	24	12.81000*	.01167	.000	12.7742	12.8458
22	24	17.76000*	.01167	.000	17.7242	17.7958
23	24	23.11000*	.01167	.000	23.0742	23.1458

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

*. The mean difference is significant at the 0.05 level.

I group for the microbial single strains, J group for control.

Multiple Comparisons

Microbial mixtures Masila crude oil

Dunnett t (2-sided)[a]

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	16	12.22000*	.00764	.000	12.1970	12.2430
2	16	32.90000*	.00764	.000	32.8770	32.9230
3	16	13.59000*	.00764	.000	13.5670	13.6130
4	16	38.33000*	.00764	.000	38.3070	38.3530
5	16	20.25000*	.00764	.000	20.2270	20.2730
6	16	9.15000*	.00764	.000	9.1270	9.1730
7	16	39.13000*	.00764	.000	39.1070	39.1530
8	16	27.09000*	.00764	.000	27.0670	27.1130
9	16	10.53000*	.00764	.000	10.5070	10.5530
10	16	8.09000*	.00764	.000	8.0670	8.1130
11	16	43.20000*	.00764	.000	43.1770	43.2230
12	16	46.48000*	.00764	.000	46.4570	46.5030
13	16	44.46000*	.00764	.000	44.4370	44.4830
14	16	42.66000*	.00764	.000	42.6370	42.6830
15	16	17.40000*	.00764	.000	17.3770	17.4230

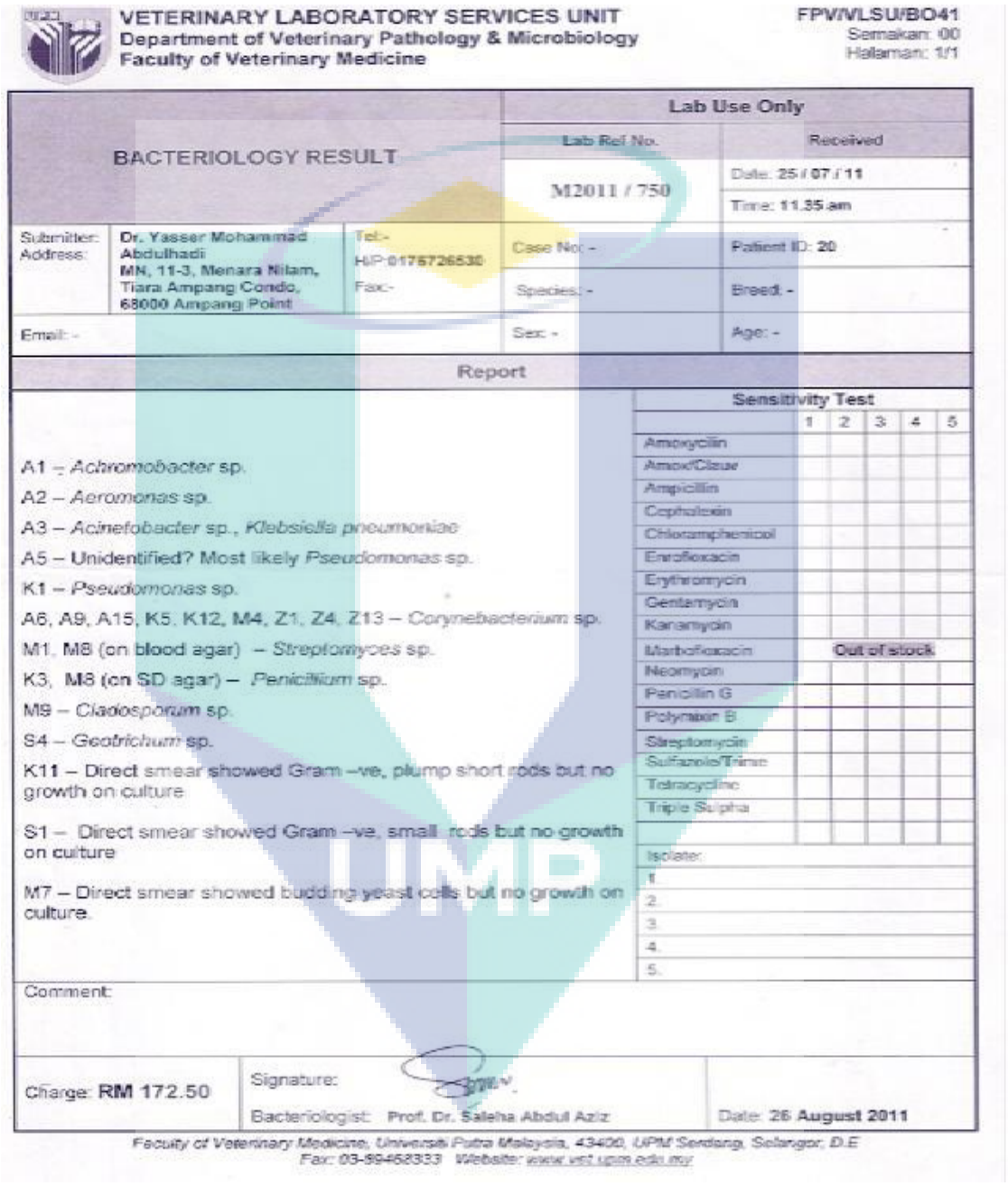
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

*. The mean difference is significant at the 0.05 level.

I group for the microbial mixtures, J group for control.

APPENDIX H

MICROBIOLOGICAL IDENTIFICATION LABORATORY RESUL



VETERINARY LABORATORY SERVICES UNIT
Department of Veterinary Pathology & Microbiology
Faculty of Veterinary Medicine

FPV/VLSU/BO41
Semakan: 00
Halaman: 1/1

BACTERIOLOGY RESULT

Lab Use Only

Lab Ref No.: M2011 / 750

Received
Date: 25 / 07 / 11
Time: 11.35 am

Submitter: Dr. Yasser Mohammad Abdulhadi
Address: MN, 11-3, Menara Nilam, Tiara Ampang Condo, 68000 Ampang Point
Tel:- H/P:0175726530
Fax:-

Case No: -
Species: -
Breed: -
Patient ID: 20
Sex: -
Age: -
Email: -

Report

A1 – *Achromobacter* sp.
A2 – *Aeromonas* sp.
A3 – *Acinetobacter* sp., *Klebsiella pneumoniae*
A5 – Unidentified? Most likely *Pseudomonas* sp.
K1 – *Pseudomonas* sp.
A6, A9, A15, K5, K12, M4, Z1, Z4, Z13 – *Corynebacterium* sp.
M1, M8 (on blood agar) – *Streptomyces* sp.
K3, M8 (on SD agar) – *Penicillium* sp.
M9 – *Cladosporium* sp.
S4 – *Geotrichum* sp.
K11 – Direct smear showed Gram –ve, plump short rods but no growth on culture
S1 – Direct smear showed Gram –ve, small rods but no growth on culture
M7 – Direct smear showed budding yeast cells but no growth on culture.

Sensitivity Test

	1	2	3	4	5
Amoxycilin					
Amox/Clavu					
Ampicillin					
Cephalexin					
Chloramphenicol					
Enrofloxacin					
Erythromycin					
Gentamycin					
Kanamycin					
Marbofloxacin	Out of stock				
Neomycin					
Penicillin G					
Polymixin B					
Streptomycin					
Sulfazole/Trimet					
Tetracycline					
Triple Sulpha					

Isolate:
1.
2.
3.
4.
5.

Comment:

Charge: RM 172.50

Signature:
Bacteriologist: Prof. Dr. Saleha Abdul Aziz

Date: 26 August 2011

Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400, UPM Serdang, Selangor, D.E
Fax: 03-89468333 Website: www.vet.upm.edu.my

APPENDIX I

1. **Khairi Ali Alennabi**, Azhari Hamid Nour, Abdurahman Hamid Nour and Yasser Mohammed Abdelhadi. 2011. Rate of Biodegradation of Crude Oil and the Controlling Factors using Microorganisms from Malaysian Environment. *International Conference on Natural Products ICNP 2011,* 14-16th November. Palm Garden Hotel, IOI Resort Putrajaya, Malaysia. Pp119

2. **Khairi Ali Alennabi**, Azhari Hamid Nour, Abdurahman Hamid Nour, Yasser Mohammed Abdelhadi. 2011. Rate of Biodegradation of crude Oil using Microorganisms from Malaysian Environment. *International Postgraduate Conference on Biotechnology. IPCB 2011,* 15-18th December. Dewan Sultan Mizan, Universiti Malaysia Terengganu. Pp122.

3. **Khairi Ali Alennabi**, Azhari Hamid Nour, Abdurahman Hamid Nour, Abdelhadi Y. M. 2011. Study of controlling factors and rate of biodegradation of used car oil by microorganisms isolated from the Malaysian soil. (In progress).

4. **Khairi Ali Alennabi**, Azhari Hamid Nour, Abdurahman Hamid Nour, Abdelhadi Y. M. 2012. Study of the rate of biodegradation of Masila crude oil versus Miri crude oil by microorganisms isolated from contaminated soil. (In progress).

5. **Khairi Ali Alennabi**, Azhari Hamid Nour, Abdurahman Hamid Nour, Abdelhadi Y. M. 2012. Study of some environmental parameters effect on biodegradation of Masila crude oil . (In progress).

PP-C28

RATE OF BIODEGRADATION OF CRUDE OIL AND THE CONTROLLING FACTORS USING MICROORGANISMS FORM MALAYSIAN ENVIRONMENT

Khairi Ali Alennabi*[1], Azhari Hamid Noor[1], Abdulahman Hamid Noor[2] and Yasser Mohammed[3]

[1] Faculty of Industry Science and Biotechnology, Universiti Malaysia Pahang, Lebuharaya Tun Razak 26300 Gammbang, Kuantan, Pahang Darul Makmur, Malaysia
[2] Faculty of Chemical Engeneering, Universiti Malaysia Pahang
[3] Faculty of Agriculture, Universiti Putra Malaysia, 43400, UPM, Serdang, Selangor Darul Ehsan, Malaysia.

Oil spills have been a major issue in industry, being hard to biodegrade. Crude oil components were found to be carcinogenic, cause respiratory problems and nervous system troubles. Bioremediation techniques for removing petroleum hydrocarbons in the soil are developed around strategies for delivering moisture, aeration and nutrients to optimize microbial activity and degradation of the pollutants. Microorganisms were isolated by selective enrichment technique. The isolation was carried out using selective medium (agar containing crude oil, used car oil and benzene) as unique carbon source. Microorganisms were identified by biochemical test. Microbial biodegradation screening was achieved in microplates containing nutrient broth, single and mixture of isolated strains and crude oil, incubated at 27 °C for four weeks, results were recorded weekly. Sterilized soil were distributed in petri dishes, contaminated with 3 % w/w crude oil, and supplemented with isolated strains and mixture of them in addition to one petri dish as a control. Petri dishes were incubated at 27 °C for four weeks. Amount of soil were extracted with toluene weekly. Absorbance was determined using spectrophotometer at 360 nm. The effects of some factors were studied such as aeration, NPK addition (nutrients) and microbial biodegraders isolated from contaminated areas. Soil was collected from UPM campus, divided in five containers. The soil was contaminated with 3 % of used car oil. Results included isolation and identification of 22 microbial species. Most of the isolated single strains as well as consortiums showed some effect on crude oil biodegradation screening. Strain A3 which was identified as Acinetobacter spp. *Klebsiella pneumonia* had the highest effect with 33 % removal of crude oil. While microbial mixture Smix was highly effective with 49 % removal. The result of biodegradation in different treatment options in time of six weeks showed that the group that was aerated had the highest removing percentage (57 %). In general, microbial consortiums were effective on crude oil biodegradation, suggesting the field treatment by microbial mixture addition, aeration and nutrient addition to achieve fast clean up results.

Malaysian Natural Products Society

UPM UNIVERSITI PUTRA MALAYSIA

CARIF

Certificate of Appreciation

This is to certify that

KHAIRI ALENNABI

has participated in the

INTERNATIONAL CONFERENCE ON NATURAL PRODUCTS 2011
METABOLOMICS: A NEW FRONTIER IN NATURAL PRODUCTS SCIENCE

14-16 November 2011
Palm Garden Hotel, IOI Resort, Putrajaya, Malaysia

as

POSTER PRESENTER

PROF. DR. MD NORDIN HJ. LAJIS
Chairman
International Conference on Natural Products 2011
(ICNP2011)
Universiti Putra Malaysia

INTERNATIONAL POSTGRADUATE CONFERENCE ON BIOTECHNOLOGY 2011

POSTER ABSTRACT

Biological and Environmental Technology

Rate of Biodegradation of Crude Oil using Microorganisms from Malaysian Environment

Khairi Ali Alennabi [*1], Azhari Hamid Noor[1], Abdulahman Hamid Noor[2], Yasser Mohammed[3]

[1]Faculty of Industry Science and Biotechnology, Universiti Malaysia Pahang, Lebuharaya Tun Razak, 26300 Gammbang, Kuantan, Pahang Darul Makmur, Malaysia,
[2]Faculty of Chemical Engeneering, Universiti Malaysia Pahang,
[3]Faculty of Agriculture, Universiti Putra Malaysia, 43400, UPM, Serdang, Selangor Darul Ehsan, Malaysia

Five soil samples were collected from car workshops areas in Malaysia. Twenty two microbial strains were isolated by selective enrichment technique. The isolation was carried out using selective medium (agar containing crude oil, used car oil and benzene) as unique carbon source. Microorganisms were identified by biochemical test and they were used in crude oil biodegradation. Microbial biodegradation screening was achieved in microplates containing nutrient broth, single and mixture of isolated strains and crude oil, incubated at 27 °C for four weeks, results were recorded weekly. Sterilized soil were distributed in petri dishes, contaminated with 3 % w/w crude oil, and supplemented with isolated strains and mixture of them in addition to one petri dish as a control. Petri dishes were incubated at 27 °C for four weeks. Amount of soil were extracted with toluene weekly. Absorbance was determined using spectrophotometer at 360 nm. The result included isolation and identification of 22 microbial species; *Achrombacter*, *Aeromonas*, *Klebsiella pneumonia*, *Pseudomonas*, *Corynebacterium*, *Penicillium*, G –ve plump short rods, *Streptomyces*, budding yeast cells, *Cladosporum* and *Geotrichum* species. Most of the isolated single strains as well as consortiums showed some effect on crude oil biodegradation screening. Strain Z13 which was identified as *Corynebacterium* spp had the highest effect with 54 % of remove of crude oil. While microbial mixture, MS was highly effective with 84 % of remove. Microbial consortiums isolated from contaminated soil exhibited a good effect on crude oil biodegradation more than the single strains. This was due to the complex compound in the crude oil which needs more microbial species to manage to degrade it. The consortiums from the same area of isolation were better due to the adaption in the same environment. Microbial mixtures can be applied as promising option in the same matter.

Keywords: **biodegradation, microorganisms, crude oil**

UNIVERSITI MALAYSIA TERENGGANU

CERTIFICATE OF APPRECIATION

Gratitude to

KHAIRI ALI ALENNABI

As participant for

INTERNATIONAL POSTGRADUATE CONFERENCE ON BIOTECHNOLOGY (IPCB) 2011

"NURTURING RESOURCES FOR A BETTER TOMORROW THROUGH BIOTECHNOLOGY"

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INSTITUTE OF MARINE BIOTECHNOLOGY
UNIVERSITI MALAYSIA TERENGGANU, MALAYSIA

Co-organized by

SOKA UNIVERSITY, JAPAN

On 15 – 18 December 2011
At Universiti Malaysia Terengganu (UMT)

PROF. DR. HIDEO YAMAMOTO
President
Soka University

PROF DATO' DR. AZIZ BIN DERAMAN
Vice Chancellor
Universiti Malaysia Terengganu