SCREENING OF FUNGI FOR DIESEL BIODEGRADATION IN LIQUID MEDIUM

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INSTITUTE OF BIOLOGICAL SCIENCE
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Name of Degree: **MASTER OF TECHNOLOGY (ENVIRONMENTAL MANAGEMENT)**

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SCREENING OF FUNGI FOR DIESEL BIODEGRADATION IN LIQUID MEDIUM

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SCREENING OF FUNGI FOR DIESEL BIODEGRADATION IN LIQUID MEDIUM

ABSTRACT

Diesel is one of the common petroleum hydrocarbon (PH) components that cause water pollution. Being as the recalcitrant pollutant, its treatment requires extensive processes which pose physically and financially challenges. Bioremediation using fungi is the emergence method that offer the solution to such challenges. This study explore the use of fungi for diesel biodegradation in liquid media. Twelve potential fungal strains have been isolated from contaminated and undisturbed soils using diesel coated agar media. Each fungal strain was further tested for their tolerance based its growth within the duration of seven days using 1% and 10% diesel concentrations (v/v) in potato dextrose broth media by the shake flask method. Five prospect fungi had been regarded to have high diesel tolerance. Simple identification method showed that they belong to the division of *Ascomycota*, and namely as, *Penicillium* sp. A*, Penicillium* sp. B, *Penicillium* sp. C, *Aureobasidium* sp. and *Aspergillus* sp. Later, the ability to degrade diesel for all five strains were assessed using redox indicator and gas chromatography mass spectrometry (GC/MS) analysis. Both assessments confirmed that all prospect fungal have the expected ability. Meanwhile, GC/MS analysis for the diesel concentration of 1% (v/v) have further narrowed down to two isolates of *Penicillium* sp.A and *Aspergillus* sp. which are to be the good diesel degrader, 70% to 90%, respectively. The rest of other isolates did not show to be a good prospect of fungi. One strain (*Aureobasidium* sp.) did not represent a good formation of biomass floc (colloidal) with the other two strains had low degradation (< 60%). However, at the diesel concentration of 10% (v/v), *Penicillium* sp.A is a better performer, when it removed almost 70% of diesel, compared to 63% removal for *Aspergillus* sp.. Final assessment was done using a higher concentration of biomass (9 g/l) with continuous stirred tank reactor. Unexpectedly, both isolates have

shown the declining of diesel reduction where, *Aspergillus* sp. reduced 41% and *Penicillium* sp.A of 77%. Nevertheless, the final assessment did not take account of factors of evaporation and headspace which may affect the overall diesel degradation assessment in a closed vessel.

Keywords: Petroleum Hydrocarbon, Bioreactor, Shake Flask, GC/MS, Redox Indicator.

PENYARINGAN KULAT BOLEH-BIOROSOT DIESEL DIDALAM MEDIUM CECAIR

ABSTRAK

Diesel adalah salah satu komponen hidrokarbon petroleum (PH) yang menyebabkan pencemaran air. Sebagai bahan cemar yang sukar diurai, rawatannya memerlukan proses yang ekstensif yang boleh memberi cabaran fizikal dan kewangan. Bioremediasi menggunakan kulat adalah kaedah yang boleh menawarkan penyelesaian kepada cabaran tersebut. Kajian ini meneroka penggunaan kulat untuk biodegradasi diesel dalam media cecair. Dua belas strain kulat yang berpotensi telah diasingkan daripada sumber tanih yang tercemar dan tidak terganggu dengan menggunakan media agar bersalut diesel. Setiap strain kulat terus diuji untuk toleransi berdasarkan tumbesaran nya dalam tempoh tujuh hari dengan menggunakan 1% dan 10% kepekatan diesel (v/v) dalam media kentang dekstros menggunakan kaedah goncang-flask. Lima prospek kulat telah dmempunyai toleransi diesel yang tinggi. Kaedah pengecaman ringkas menunjukkan bahawa mereka tergolong dalam divisi Ascomycota, dan iaitu, *Penicillium* sp. A, *Penicillium* sp. B, *Penicillium* sp. C, *Aureobasidium* sp. dan *Aspergillus* sp. Kemudian, keupayaan untuk merungkai diesel untuk semua lima strain dinilai melalui penunjuk redoks dan analisis spektrometri jisim kromatografi gas (GC /MS). Kedua penilaian tersebut mengesahkan bahawa semua prospek kulat mempunyai keupayaan yang diharapkan. Sementara itu, analisis GC / MS untuk kepekatan diesel sebanyak 1% (v / v) telah menjurus kepada dua isolat *Penicillium* sp.A dan *Aspergillus* sp. yang dapat merungkai diesel dengon baik, masing-masing tesoh mengurangkan 70% hingga 90%. Bagi tiga isolat yang lain, iait satu strain menunjukkan pembentukan biojisim flok dalam bentuk koloid dan dua lagi strain

lain mempunyai kemerosotan diesel yang rendah (<60%). Walau bagaimanapun, pada kepekatan diesel sebanyak 10% (v/v), *Penicillium* sp.A telah berupaya merungkai hampir 70% daripada diesel, berbanding 63% untuk *Aspergillus* sp. Penilaian akhir dilakukan dengan menggunakan lebih tinggi kepekatan biojisim (9 g / l) dengan tangki reaktor yang dikocak secara berterusan. Walaubagaimana pun, kedua-dua isolat telah menunjukkan penurunan darjah penguraian diesel yang, *Aspergillus* sp. berkurang menjadi 41% manakala *Penicillium* sp.A sebanyak 77%. Walau bagaimanapun, penilaian akhir ini tidak mengambil kira faktor penyejatan dan kekosongan ruang-atas reaktor yang boleh menjejaskan pengurangan diesel secara keseluruhan di dalam tangki yang tertutup.

Kata kunci: Hidrokarbon Petroleum, Bioreaktor, Goncang Flask, GC/MS, Penunjuk Redoks.

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Hanieh Mofidi

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LIST OF SYMBOLS AND ABBREVIATIONS

<	:	More than
>	:	Less than
%	:	Percentage
BTEX	:	Refers to the benzene, toluene, ethylbenzene and xylene
^{o}C	:	Degree Celsius
cm	:	Centimeter
D	:	Diesel
DNA	:	Deoxyribonucleic Acid
DTi	:	Diesel tolerance index
F	:	F statistic
F crit	:	F critical value
FGI	:	Fungal biomass growth inhibition
F-test	:	Fisher's test
F-X	:	Fungal code
g	:	gram
GC/MS	:	Gas Chromatography Mass spectrometry
Hg	:	Inch of mercury (unit of measurement for pressure)
L	:	Litter
Lac	:	Laccase
LiP	:	Lignin peroxidase
mg	:	Milligram
Min	:	Minute
µl	:	Microliter
ml	:	Milliliter

mM	:	Micromole
mm	:	Millimeter
MnP	:	Manganese peroxidase
NEPCO	:	New England Petroleum Corporation
PAH	:	Polycyclic Aromatic Hydrocarbon
PCR	:	Polymerase Chain Reaction
PDA	:	Potato Dextrose Agar
PDB	:	Potato Dextrose Broth
PH	:	Petroleum Hydrocarbon
pH	:	potential of Hydrogen
P-value	:	Probability value
RB		Round-bottom
rpm	:	Rotation per minute
RT	:	Retention time
TCA	:	Tricarboxylic acid cycle (kerb cycle)
T-test	:	Turkey ‘s test
v	:	Volume
U/ml	:	Units per milliliter
U.S. EPA	:	United States Environmental Protection Agency
UV	:	Ultraviolet
WRF	:	White rot fungi
WT	:	Weight

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background

Hydrocarbon fluid, as a major energy sources, release into the environment whether unintentionally or due to human diligence is a main cause of pollution to air, fresh water (surface water and groundwater), marine environment and soil (Edwan Kardena, 2015). Regularly during the oil and gas exploration, production, transportation, and storage approximately five million tonnes of hydrocarbons in the forms of crude oil, refined oil, diesel, gasoline and petrol enter the aquatic and terrestrial systems anthropogenically each year, and contaminated ecosystem around the world (Chaudhry *et al.*, 2012; Das & Chandran, 2011).

Water pollution by petroleum hydrocarbons (PH) such as diesel is one of the momentous environmental challenges, as they are lipophilic and more toxic than the other petroleum products due to their higher volume of light weight hydrocarbons (Weiner, 2012). Diesel can alter the ecosystem and cause deleterious effects to aquatic fauna and flora and, eventually harm the human being (Ameen *et al.,* 2016; Behnood *et al.*, 2014). Moreover contamination with PH would cause extensive devastation of adjoining system accumulation of persistance pollutants in animals and plant tissues as they may bring death or transformation (Kadri *et al*., 2017; Peng *et al*., 2008). Some of PH compounds persist in the water bodies and, due to their hydrophobicity, become associated with particulate matter, such as clays and humics that are deposited in soils and sediments (Yu *et al*., 2015). Removing these organopollutants from the water in an ecologically responsible, safe, and cost-effective way is an urgent concern. Hence, there is enormous curiosity to make use of biodegradation technologies using various microorganisms for removing PH due to their environmental safety and comparatively at lower cost against current physical (absorption) and chemical (redox reaction) treatment approaches (AI-Jawhari, 2014).

Various biodegradation activities execution of many natural microorganisms has been studied for several last decades; as follow supplementary on bacteria, and fungi in lesser degree (Chaudhry *et al*., 2012; Rosas, 2014; Pinedo-Rivilla *et al.*, 2009). Superiority of using fungi over bacteria is their capability to grow faster on a large spectrum of substrates in huge biomass and at the same time, their extracellular hydraulic and non-specific enzymes activities which can mineralized intensively a number of various organic pollutants and/or break them down into less toxic metabolites rather than their parent substances (Chan, 2011; Kadri *et al*., 2017; Maddela *et al.*, 2015). Additionally fungal implementation in extreme environmental conditions and their lower race of contamination than bacteria, make them more efficient to biodegradation process (Anderson, 2016).

Many native strains of fungi have been reported with great potential for remediation of PH (Chaudhry *et al*., 2012). This statement has been reported by several studies of filamentous fungi, e.g. *Aspergillus, Amorphoteca, Fusarium, Penicillium*, ability to be as a good degrader agents (Varjani, 2017; Marchand *et al.*, 2017). However, Grosberg *et al*. (2012) have been reported the biodiversity for fungi species in marine and fresh water equal to <1000 species for each environment; whereas for terrestrial species of fungi has been amounted more than a million. Specifically as a saprotrophs, fungal hyphae always tends to attach and grow on solid surfaces, e.g. sediments and soils, invade substrates, and plant and animal tissues; as a result, their existence as the free-floating microbiota in water columns can be impossible (Dighton & White, 2017). Therefore, extraction of contaminated soil indigenous fungi in a certain extent is simple and lucid rather than from contaminated water. Definitely, the rate of bioremediation of pollutant by fungi depends on the environmental conditions, type of microorganism spices, as well as the nature and structure of the compound to be removed as a contaminants (Kadri *et al*., 2017).

1.2 Problem Statement

Physical technologies commonly used for the PH remediation including mechanical, burying, evaporation, dispersion, washing, be proven efficient to treat similar compound of diesel in water (Tansel *et al.*, 1995), however, they are expensive, with adverse environmental outcomes (Das & Chandran, 2011). Moreover, due to the significance of ongoing PH pollution toxicity in aquatic system with the absence of a reasonable settlement, and ecologically chargeable method of clean-up is extensively required. One growing mechanism of PH decontamination of water that may fit mentioned requirements is the bioremediation. Use of fungi in bioremediation has been exploited and making the science of it, less known. This study focus on the use of fungi to investigate the full potential of PH remediation including finding new isolates.

We hypothesize that experimental applications of indigenous soil fungi degradation will lead to reduction of petroleum hydrocarbon in liquid medium with the use of non-destructive treatment method.

1.3 Importance of This Study

When the study is to be completed, more significant findings will be obtaining more efficient soil fungi as a diesel hydrocarbon, degradation/ deterioration agent.

1.4 Aim and Objectives

This research is aiming for a practical solution with regards to scientific discovery of using biotechnological advancement to treating the petroleum hydrocarbon threat to the environment. This study explores the potential of using indigenous fungi to neutralize such threat. The intended aim of this study is to be achieved by the following research objectives.

Objectives of Research

1. To screen and isolate fungi from various soil sources for diesel biodegradation in liquid medium.
2. To explore the ability of selected fungi to treat of contaminated liquid medium with petroleum hydrocarbon.
3. To examine the biodegradation of diesel using high concentration of biomass in bioreactor system.

1.5 Expected Outcome

Due to sustainability and environmental friendly of natural indigenous soil fungal may can use as a degradation agent for either in-situ and ex-situ or pilot scale and in-vitro in diesel contaminated aquifers.

1.6 Scope of the Work

The main scope of this study was fungi isolation from contaminated and natural soil for screening of indigenous fungi potential of diesel biodegradation in liquid media using qualitative analysis. This study will not pursue the identification of the isolates, therefore, we will not know whether is new or similar to other studies.

CHAPTER 2: LITERATURE REVIEW

2.1 Water Contamination by Diesel

Anthropogenic activates such as pipeline or facilities storage leakage, undesired accidental spillage from vehicles and vessels, deliberated disposal of excess fuel, industrial effluents and municipal runoffs are some examples that can cause entering of petroleum hydrocarbons contamination to surrounding environment, i.e., soil, surface, underground and coastal waters, and ocean (Ameen *et al*., 2016; Varjani, 2017). It is important to bear in mind that oil motion is different in various environmental spillage area. Fuel oil is more static and move down slowly, when the spillage occurs on terrestrial ecosystems. Meanwhile, if it takes place in the open water reservoirs, because of the tidal influences, this movement becomes more extensive in the wider space and time (Speight & El-Gendy, 2017). Accordingly, the environmental risk and impact will increase while the recovery potentials decline (Figure 2.1). It is reported by National Research Council that approximately 1.3 tons of petroleum products totally enter to the oceans from all sources annually (Hassanshahian & Cappello, 2013).

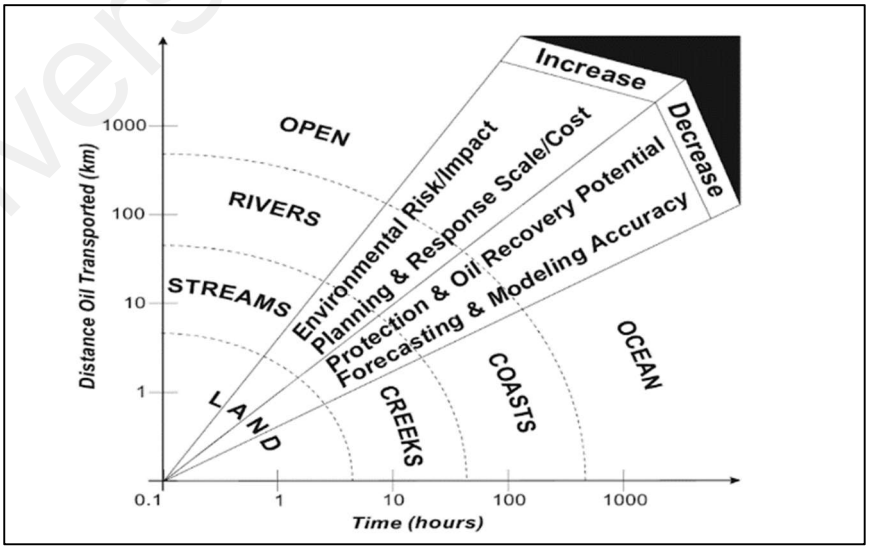


Figure 2.1: Oil spills movement in the frame of the time and space in different ecosystems (adopted from Fingas, 2016).

Oil spills can damage the flora and fauna in the coastal area and subtidal sediments. It can also contributes to oxygen deficiency in marine sediments devastating microbiota diversities. Therefore, for better understanding of bioremediation strategies should be taken, it is vital firstly realize the petroleum oil properties, environmental distress, the fate of fuel oil in that site, as well as, degradation pathway of such a pollution in the ecosystems, and the factors that may affect the feasibility of this degradation (Varjani, 2017).

2.1.1 Diesel Fuel Fate in Marine and Fresh Water

Specifically, with increasing demand for petroleum oil day by day and transporting more than half of these products across the world by ocean and seas as the supreme sink for PH pollutants, the accidental spillage of these kind of pollutants into water is vulnerable and will grow significantly (Xue *et al*., 2015). Yet, literatures on oils spills and its impacts on large inland rivers are unexpectedly limited. Although, there are some exceptional in the impact of the oil spill in a large rivers and open marines. In general, diesel is a very light oil with low density- still is much higher than water volumetric mass density- would float in both salty sea waters and freshwater rivers.

Chemical, physical and biological transformation recalled as weathering processes in the aquatic ambient occur once the petroleum refined products enter the water. Weathering processes may influence chemical, physical and biological characteristic of the initial pollutants composition as well as environmental impacts (Board *et al.*, 2003). These processes are including volatilization, photo-chemical oxidation, dissolution, emulsification, adsorption, spreading, advection, dispersion, partitioning, sedimentation as well as ingestion and natural degradation by microorganisms (Fingas, 2016). Total movement of organic pollutants, such as diesel, in the aquatic environment and factors may affect the rate of that action are detailed in Table 2.1.

Table 2.1: Fate of organic contaminants (diesel) in the aquatic environment (Wick *et al*., 2011).

Process	Consequences	Factors
Transfer *(processes without altering the structure)*		
Volatilization	Loss of mass load due to evaporation	Vapor pressure, wind speed, temperature, radiation intensity, water state, surface area.
Absorption	Uptake by plant roots or organisms ingestion	Contact time, cell membrane shift, organism's type, susceptibility.
Degradation *(processes that alter the structure)*		
Biological	Degradation by microorganisms, bio-degradation/ co-metabolism	Environmental factor (pH, temperature, oxygen), nutrient content, contamination composition, bioavailability, molecular weight of component, microbial population.
Chemical	Alteration by chemical processes, e.g. photochemical and oxidation- reduction responses.	pH, component structure, sunlight UV power and duration, other elements similar to biodegradation
Sequestration *(processes that relocate components into long-term storage without alteration)*		
Adsorption	Removal through interaction with sediments	Organic matter content
Diffusion	Dispersion and spreading into the sediments and soil micro-pores and mixing with waves.	Hydrophobicity of micro-pores and diesel components
dissolution	Dissolving into the water column	Structure and polarity of diesel components, kinetic condition,

Generally, when fuel oil encounters to the surface aquatic reservoirs, e.g. oceans, lakes and fresh waters, some of chemicals evaporate into the atmosphere and/or are stored in water column. Stored compounds might find in the form of dissolved in water or sink down slowly into the sediments after attached to the particulate matters (Wick *et al.*, 2011).

Chemicals evaporation could happen with breaking down of components by sunlight interaction as a photooxydation process, or react with other atmospheric chemicals (Risher, 1995). Evaporation process is usually the most predominant process happening during first 48 hours of the fuel spillage into the water. This process is responsible to volatilize the low to medium molecular compounds with low boiling points and low vapour pressure into the atmosphere. The feasibility of this procedure depends on pollutant composition and its physical properties, surface area, wind power, temperature of the water and air, solar radiation intensity and ocean state. Based on the Figure 2.2 more than 40% of diesel oil will vanished during vaporization process after 40 hours at 15 ^{o}C degree (Board *et al*., 2003).

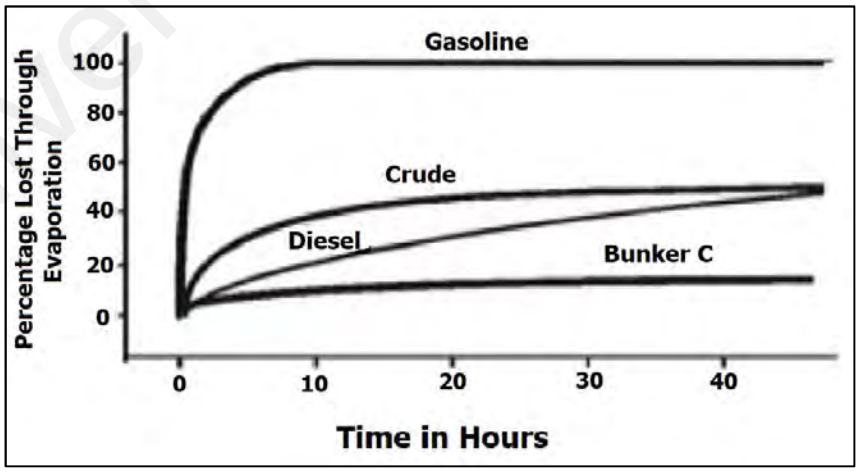


Figure 2.2: Volatilization rate with four different types of oil (adopted from Board *et al.,* 2003).

Dissolved components may also up taking by organisms, e.g. bacteria, algae, and fungi, and broken down structurally and/or trapped in body tissues (Walker *et al*., 2005). Relying on variety factors, breaking down of chemicals by native microbial residences may take place up to one year. Thus, due to the toxicity of these water-soluble diesel fractions for aquatic organisms (involving all other fractions utilizing microorganisms), and its great impression to the ecosystem as one of that cause the pollution mass load, dissolution as one of the important fate for hydrocarbons also should keep in consideration equally (Hassanshahian & Cappello, 2013). Figure 2.3 represents the total petroleum oil fate in marine environment according to study by Hassanshahian and Cappello (2013) with minor amendments which were discussed earlier in details in the table 2.1.

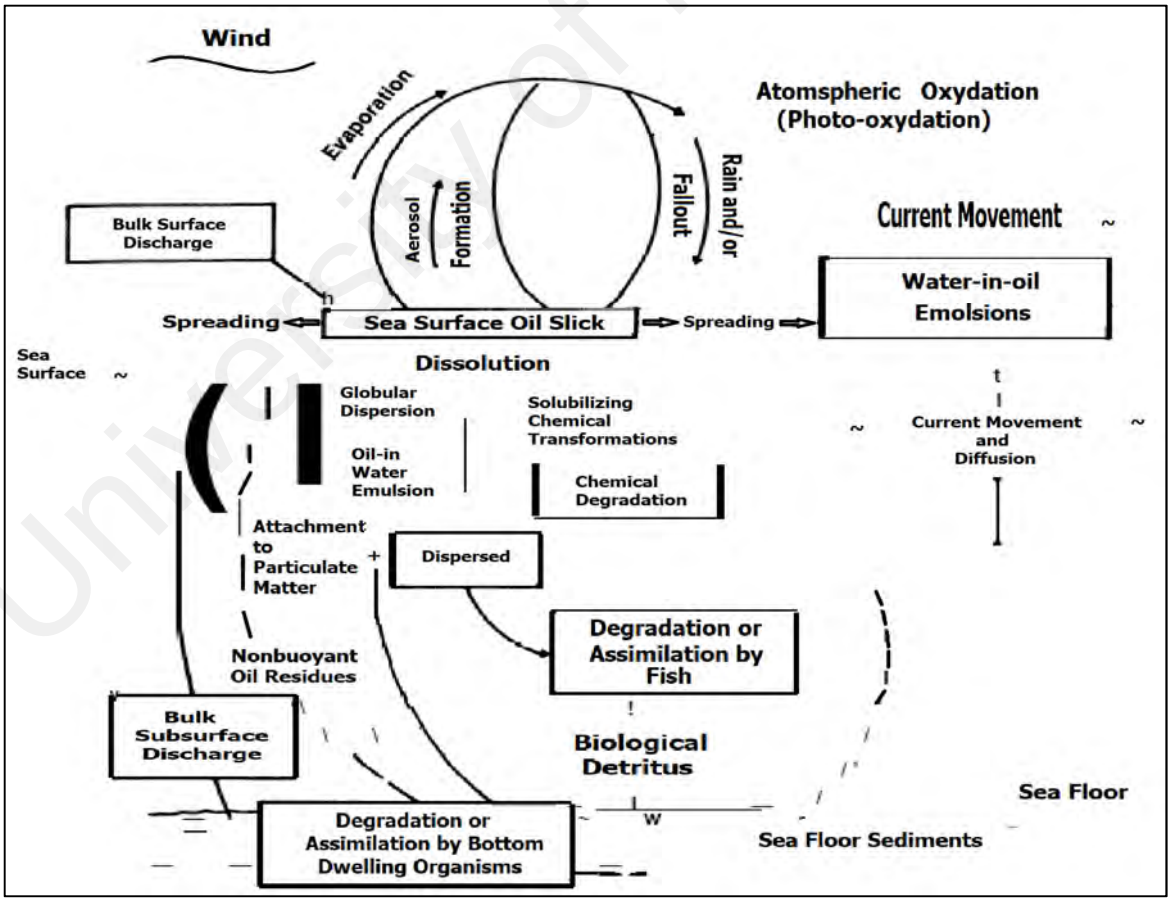


Figure 2.3: Schematic diagram of petroleum oil fate in marine restoration (adopted from Hassanshahian & Cappello, 2013).

However, diesel fate in the fresh waters such as river is resulting in some exceptions to that in marine reservoir. In the large muddy rivers, which carrying huge amounts of sediments and bulks in their currents, there is high potent of absorption of oil droplets and eventually make them sink-down to the bottom of the river. In other hands, if either sedimentation not happened or the clean-up application not enough, usually oils flow down the streams to the mouth of a river where it links to ocean. The NEPCO-140 oil spill of 1976 in the St. Lowrence River of Thousand-island area resulted in devastating damage to wetlands' plants and extensive oiling of marshes is one of the historical file of unintentional spillage into freshwaters (Vandermeulen & Hrudey, 1987). The clean-up technologies and strategies will be different and critical for the river banks with plants surroundings than for that with open sandy or rocky seashores of coasts (Fingas, 2016).

2.2 Diesel Characteristic

Petroleum oils are usually a sticky liquid with a variety complex mixture of hydrocarbons consisting of mainly carbon and hydrogen atoms and small amount of heteroatomic compounds, i.e., nitrogen, sulphur, and oxygen as well as a trace elements of heavy metals (Risher, 1995; Logeshwaran *et al*., 2018). Chemical structures of compounds to be found with different molecular weights ranging from light, volatile, linear short-chained to weighty, long-chained, splitter and stable organic compounds. They are diverge depending upon to derived crude oil sources and implemented refinery process of product generation. After refining process modification such as condensation, polymerization, alkylation and cracking procedure, different crude oil fraction are altered to high-valued commercial and industrial products acting as naphtha, gasoline, jet fuel (kerosene) and diesel fuel oils (Speight, 2005). Several grades of diesel fuel are designated in order of distinct viscosity and density properties, with slightly difference in chemical composition balance. Nevertheless, all kinds consist of heterogeneous combination of hydrocarbons (Chevron Global Marketing, 2007).

2.2.1 General Properties

Among other kinds of petroleum fuels, diesel with 43% of thermal efficiency is the most common and useful engine oil. Typical diesel is non-polar, appearance range from pinkish to pale yellow, absorption tendency to solid organic matters properties with poor water solubility and generally kerosene-like odor. All kinds of diesel fuel with slight difference in chemical component structure are liquid in room temperature, although they are able to evaporate with high susceptibility and flammability (Risher, 1995). Petrodiesel normally freezes around temperature of -8 ^{o}C with high boiling temperature (180-360 ^{o}C), which is increasing with increases of carbon number and low vapour pressure (Chevron Global Marketing, 2007).

Some chemical characteristics of diesel fuel's hydrocarbon components are illustrated in Table 2.2 in accordance with hydrocarbon classes. In accordance to water solubility values for different constituents in the diesel fuel, diesel can dissolved in water partially and as a consequence, it can accumulate in microorganisms' tissue. However, solubility in water depends on temperature, pH, salinity, and organic compounds concentration. The greatest quantity of diesel's constituents have high vapour pressure, therefore, can easily evaporate. This is important for risk establishment related to excavating sediments (dredging), field sampling and surely for lab safety (Wick *et al.,* 2011).

Table 2.2: Physical properties for selected hydrocarbons class (Kauppi, 2011; Kim *et al*., 2015).

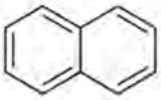
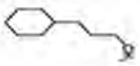
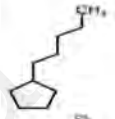
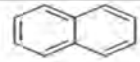
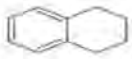
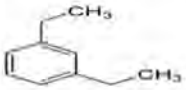
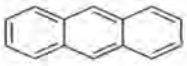
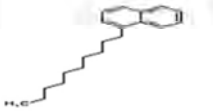
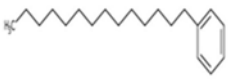
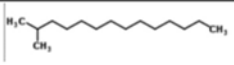
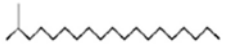
Hydrocarbon Class	Compound	Formula	Density (20°C, g/cm^2)	Water solubility (mg/L)	Vapour pressure (pa at 25 °C)	Boiling point (°C)	Freezing point (°C)
n-Paraffin	n-Decane	$C_{10}H_{22}$	0.7301	0.052	195	174	-30
	n-Pentadecane	$C_{15}H_{32}$	0.7684	4×10^{-5}	457×10^{6}	271	10
	Eicosane	$C_{20}H_{42}$	0.7843	1.9×10^{-3}	0.0186	344	36
Naphthene	n-Butylcyclohexane	$C_{10}H_{20}$	0.7992	-	386.63	181	-75
Aromatic	(a)Benzene	C_6H_6	0.8765	1780	12.7×10^{3}	80.1	5.5
	Naphthalene	$C_{10}H_8$	1.175	31	10.93	218	80
	Anthracene	$C_{14}H_{10}$	1.251	8	0.08	341	215

2.2.2 Chemical Composition

Diesel with common average chemicals formula approximately ranging from $C_{10}H_8$ to $C_{20}H_{42}$, Predominantly consists of about 64% saturated hydrocarbons (mostly aliphatic straight long-chained compounds of alkanes primary n, iso-paraffins and cycloparaffins), 1-2% unsaturated alkenes (olefins), 20-35% aromatic hydrocarbons (including alkylbenzenes and 2-, 3-ring aromatics such as naphthalene with 5 or 6 carbon per a ring), ranging from 0.4 WT % to 2.4 WT % of sulphur, and lower than 100 ppm of nitrogen content, as well as minor amount of oxygen atom (Anderson, 2016; Risher, 1995). Table 2.3 represents the commonly diesel hydrocarbons substitutes with typical structures. Components relative existence in the diesel fuel oil depend on feedstock supplied corresponding to meet the requirements of demands, climate condition, and season, as well as, processing strategies (Chevron Global Marketing, 2007; Speight, 2005).

Along with most predominant hydrocarbon atoms, diesel fuel also consists of very small quantities of metals, e.g. vanadium and nickel and variety of additives such as amines, phenols and polymeric substances due to better ignition performances (Logeshwaran *et al*., 2018; Kauppi, 2011). Heavy residual of distillation process and thermal cracks material might find in very nominal quantity in the diesel fuel profile (Speight & El-Gendy, 2017).

Table 2.3: Probable chemical structure of diesel hydrocarbons (Chevron Global Marketing, 2007).

Hydrocarbon Class	Compound	Chemical Formula	Molecular Structure
Naphthene 	cis-Decalin	$C_{10}H_{18}$	
	n-Butylcyclohexane	$C_{10}H_{20}$	
	n- Pentylcyclopentane	$C_{10}H_{20}$	
	n-Nonylcyclohexane	$C_{15}H_{30}$	
	n-Decylcyclopentane	$C_{15}H_{30}$	
	n-Tatradecylcyclohexane	$C_{20}H_{40}$	-
	n-Pentadecyclcyclopentane	$C_{20}H_{40}$	-
n-Paraffin (n-alkane) 	Decane	$C_{10}H_{22}$	
	n-Pentadecane	$C_{15}H_{32}$	
	Eicosane	$C_{20}H_{42}$	
Aromatic (benzene) 	Naphthalene	$C_{10}H_8$	
	Tetralin	$C_{10}H_{12}$	
	1,3-Diethylbenzene	$C_{10}H_{14}$	
	Anthracene	$C_{14}H_{10}$	
	1-Penthylnaphthalene	$C_{15}H_{18}$	
	1-Decylnaphthalene	$C_{20}H_{28}$	
	n-Tetradecylbenzene	$C_{20}H_{34}$	
Iso-Paraffin (Iso-alkane) 	2-Methyltetradecane	$C_{15}H_{32}$	
	2-Methylnonadecane	$C_{20}H_{42}$	

2.2.3 Toxicity of Compounds

Although, the toxicity of the petroleum hydrocarbons usually expands with increases in molecular weight; but petroleum products with lower molecular weight (LMW) are considered to be more toxic in aquatic ecosystem (Logeshwaran, 2018). This is because of their highly mobile in the aquatic system; due to its lower evaporation point and easier to be absorbed (Ameen *et al*., 2016). The LMW (such as short-chained n-alkanes) will vaporise from the surface and enter the atmosphere. However, the higher molecular weights (HMW) (i.e. more longer chained and branched aliphatics and aromatics) with low water solubility possession will stand on the aquatic ambient, and resulting in absorption by particular matter and sediments (Kauppi, 2011; Risher, 1995). Nevertheless, toxicity impacts of diesel fuel is unique in each spillage. This impact is based on the environmental circumstances during and after the time of spillage, biota diversity at the risk area, the pollution load and commercial composition of diesel fuel. Therefore, the quality and quantity of common diesel chemical molecules could be vary when encountering to the environment. Additionally, weathering processes which transforming the initials into the mix variety of structures and characteristics would give different toxicity applications (Fingas, 2016).

Spreading fuel oil over the water surface lead to oxygen depletion, pH changes, and under-water plants devaluation in the marine environment extensively (Cripps & Shears, 1997). Furthermore, petroleum hydrocarbon as diesel fuel, whether directly or indirectly in the form of dissolved constituents, adsorbed components on to the particulate matter as the food in micro-droplet particles to be taken up by aquatic living creatures (Vandermeulen & Hrudey, 1987). Petroleum derivatives such as diesel oil, that accumulate in the organisms' tissue can be bio-amplified in the food chain, and can cause broad and everlasting damages. Dispersing, dissolving, light blocking, and emulsifying of fuel oil spillage into the water bodies can cause meteorological effects on aquatic

environment (Risher, 1995). Oil spillage can cause the difficulties to the birds and mammals when their piloerection are reduced, and affect their buoyancy and insulation (Fingas, 2016). For instance, approximately 6×10^{5} L of diesel oil released in over 100 km^2 at Antarctica sea water caused 300 death of mostly intertidal limpets (50% population reduced) a day after spillage (Cripps & Shears, 1997). Moreover, the concentration for n-alkanes and PAHs were remained relatively in high level in the cell tissues of survivals close to the spillage station.

Most toxic parts of diesel compounds belong to long-chained aliphatic and aromatic hydrocarbon fractions which persist in environment and also resist on biodegradation. According to U.S. EPA, naphthalene, anthracene, and pyrene (aromatics) with 2-, 3- and 4-benzene rings are categorized as priority pollutants because of their toxicity perspectives. Moreover, such compound can enter the food web by microorganism uptake in the sediment, which eventually to be accumulated at higher trophic level. Such can be observed, when these compounds found to be accumulated in human tissues (Wick *et al*., 2011). Cyclic aromatics can easily absorbed by digestional tract and localize in the body fat because of its favourable lipid-soluble characteristics (Abdel-Shafy & Mansour, 2016). In addition, due to the relatively high solubility and volatility, monocyclic aromatic hydrocarbons such as benzene in diesel (one of the BTEX compound) can act as neurotoxin and carcinogen (Fingas, 2016). These expected toxicity effects of fuel oil spill could amplify in the tropical condition. Common features such as high temperature and intensive sunlight power will accelerate the vaporization and photooxydation processes. Therefore, not only volatile fractions will vaporize and enter into the atmosphere rapidly, but also intensive UV radiation will impose the higher energy to break the bonds of aromatic hydrocarbons; resulting in production of oxygen free radicals which can extremely damage the cells of bio-tissues (Fingas, 2016).

Diesel fuel discharges in the aquatics could imbalance marine natural cycles and food chain. Additionally, flying impairment in birds and reproductive disability in fish are also reported in the water contaminated site by petroleum hydrocarbons (Singh & Chandra, 2014). Human kinds also can be exposed to adverse effects of diesel compounds through inhalation, ingestion, oral, or direct dermal contacts (Abdel-Shafy & Mansour, 2016). Tumorigenesis, irritation, arrhythmia and depression are numbers of health responses for thermal crack materials and additives existence in diesel fuel (Kauppi, 2011). At the worse cases, diesel could poison, kill and mutate when entering microorganisms, animals, and human body (Ameen *et al*., 2016; Xue *et al.*, 2015). Finally, coastal wetlands, tourism and marine source industries (fishery, coastal salt firms) and on top of those, economics can affected by fuel oil spillage and contamination in marine environment.

In respective to all above toxic impacts, it is prerequisites for understanding the expected fates as well as biodegradation pathway of such a pollutant in aquatic ecosystems (Icgen & Yilmaz, 2017). Regarding to what we state, due to toxicity of petroleum hydrocarbons which are carrying high amounts of diesel components, some removing actions should be done from the aquatic ambient.

2.3 The Use of Fungi to Remediate Petroleum Hydrocarbon

Petroleum fuels have adverse effects on the plants, animal's ecosystems and humans. Though it may seem strange, with small amount of petroleum discharges into the aquifers the quantity of its dissolved components could take over the maximum contaminant limits (Okoh, 2006). In order to control and eliminate the toxic impacts of these compounds from ecosystem, the treatment and clean-up strategies should be taken.

The remediation techniques of oil polluted site are categorized into three methods of physical, chemical, and biological. Variety of chemical and physical methods are being used to dispose and remediate the exposed site to the petroleum fuels. Adsorption,

skimming, dispersants, booming floating oil, absorption, combustion promoters, in-situ burning, gelling agent, solidification and mechanical system have being implemented in a way of toxicity reduction of PH in water (Risher, 1995; Xue *et al.*, 2015). For example, incorporating natural sorbents such as cotton wool, rice straw, kenaf, and many more in aquatic oil spillage have been investigated intensively for their high absorption tendency. Though, they can also absorb water, and thereby stimulated to sink down (Jain *et al*., 2011). However, generally physio-chemical methods should be captivated in a serious rapid recovery drifts (Das & Chandran, 2011). Although, in respect of inflexible and inefficient contaminant degradation of these conventional technologies, as well as being shifted contamination from one site to another (e.g. water to atmosphere), they are not ecological refurbishment (Das & Chandran, 2011; Xue *et al*., 2015). Additionally, in the case of large spills even most implemented responders such as skimmers and adsorbent are neither rarely functional, nor very successful (Hassanshahian & Cappello, 2013).

In contrast, comparing with other clean-up methods, bioremediation/biodegradation process by employment of appropriate natural microorganisms is the promising technique for contaminated area (Koyani, 2011). Bioremediation is one of the most cost- effective and eco- friendly method in sense of that can be done on the site without involving excavation (Al-Hawash, 2018). Interest in bioremediation has grown bigger, after successful bioremediation clean-up in Exxon Valdes oil spill of 1989 in aquatic ambient (Okoh, 2006). Besides, in terms of actual biodegradation, it is promising approach in detoxification, complete decomposition and biotransformation of initial pollutant substances into harmless inorganic compounds (Varjani, 2017; Xue *et al*., 2015). These by-products such as CO_2, H_2O (aerobic) or CH_4 (anaerobic) and generation of massive cultural biomass without adverse effect on ecosystem are the results of biotransformation processes through microbial community (Abdel-Shafy & Mansour, 2016). The induction in microbial population would continue as long as the contamination sources are

available, and after completion of degradation process it is naturally decline (Singh & Chandra, 2014). Koyani (2011) reviewed that microorganisms are owning the capacity to degrade variety mixture of hazardous organic compounds including petroleum fuels either endogenous or exogenous in the environment. The basic principle of the PH biodegradation process by fungi is illustrated in Subsection 2.3.2, Figure 2.6.

Biodegradation of petroleum fuels substances can be achieved by oleophilic microorganisms such as bacteria, fungi, yeast, viruses, protozoa and algae either as distinct culture or consortium of microbial community (Varjani, 2017). Bioremediation of petroleum polluted sites have been studied intensively through two main methods of bioaugmentation and biostimulation (Singh & Chandra, 2014) under controlled laboratory conditions and field trials (Okoh, 2006). Biostimulation is the process of adding stimulators, e.g. nutrients into the contaminated site for enhancing the growth and metabolisms of the native attenuations to degrade the target pollution. Bioaugmentation is the process of affixing specific microorganisms or genetically adapted microbes into the polluted area for degradation of particular contaminants as well as make detoxification process feasible (Singh & Chandra, 2014).

Several studies have proved that there are natural plenty distributed petroleum hydrocarbon-utilizing organisms in the environment (Ma, 1998). Though investigating and using indigenous microorganisms through augmentation is the leading solution to microbial enhanced petrol oil recovery (Dhar *et al.*, 2014; Gargouri *et al.*, 2015; Gupta, 2012; Marchand *et al.*, 2017; Yu *et al*., 2015). Xue *et al*. (2015) have reviewed that there are more than 100 Genera and 200 species of PH degrader microorganisms including bacteria (*Archromobacter, Acinetobacter, Alcaligenes* and more), cyanobacteria, fungi (*Fusarium, Penicillium* and *Aspergillus*) and algae in the aquatic restoration. However,

along with the other degrader microorganisms, fungi are also important decomposers of organic matters in ecological systems.

Greater PH biodegrability of fungi than bacteria has been reported by Al-Nasrawi (2012). Although, fungal species are not organic pollutant faster degrader compared to bacteria, but with wide variety range of petroleum oil-utilizing classification have low/non-specific catabolic enzymes in xenobiotic mineralization (Kadri *et al.,* 2017). In addition, extended mycelia network growth in the short-term duration compared to the bacteria, independent utilization of pollutant as a carbon and energy source, and well performance under limitation of external parameters make them well suited for exploiting in bioremediation process (Koyani, 2011; Lahkar & Deka, 2016).

2.3.1 Fungal Sources and Petroleum Hydrocarbon Degradation Potential

Fungi bioremediation process are known as mycoremediation. Natural population of fungi were used to detoxify of crusty organic pollutants into simple organic compounds to gain carbon and energy (Das & Chandran, 2011). Fungal are any either single-celled or multinucleate heterotrophs that classified in the group of eukaryotic organisms which includes microorganisms such as yeast and mods, as well as the most well-known mushrooms. Fungi are found in various living and non-living objects, inside the bodies of plants and animals, and moving through the air as spores (Evans & Hedger, 2001; Kues, 2015; Norton, 2012). They are mostly in terrestrials and in less extent have adjusted to marine and fresh water and even relatively not occurring very often in deep-ocean terrain (Bass *et al*., 2007; Falkiewicz-Dulik *et al.*, 2015; Hassanshahian & Cappello, 2013). However, biodiversity and bioavailability of these microorganisms in massive marine arena to mitigate PH contamination depends upon local environment condition and other biotic and abiotic elements such as pollutant compatibility which differ for each site and microbial species (Figure 2.4).

Detecting the proper fungus species with corresponding properties is crucial for biodegradation effectiveness. Broad range of native fungi species have been classified and studied as PH decomposer in the environment from different variety origins and have been used for bioremediation purpose since last century (Xue *et al*., 2015). However, *Ascomycota* is the largest phylum of indigenous fungi containing 1.5 million species. This division is the most important and reported kingdom from the point of PH biodegradation (Falkiewicz-Dulik *et al*., 2015). It is in agreement with the review study by Harms *et al*. (2011) which classified 88 species of sub-phyla *Ascomycota* as the major and diverse organic pollutants, e.g. alkanes, diesel, PAHs and crude oil degrader.

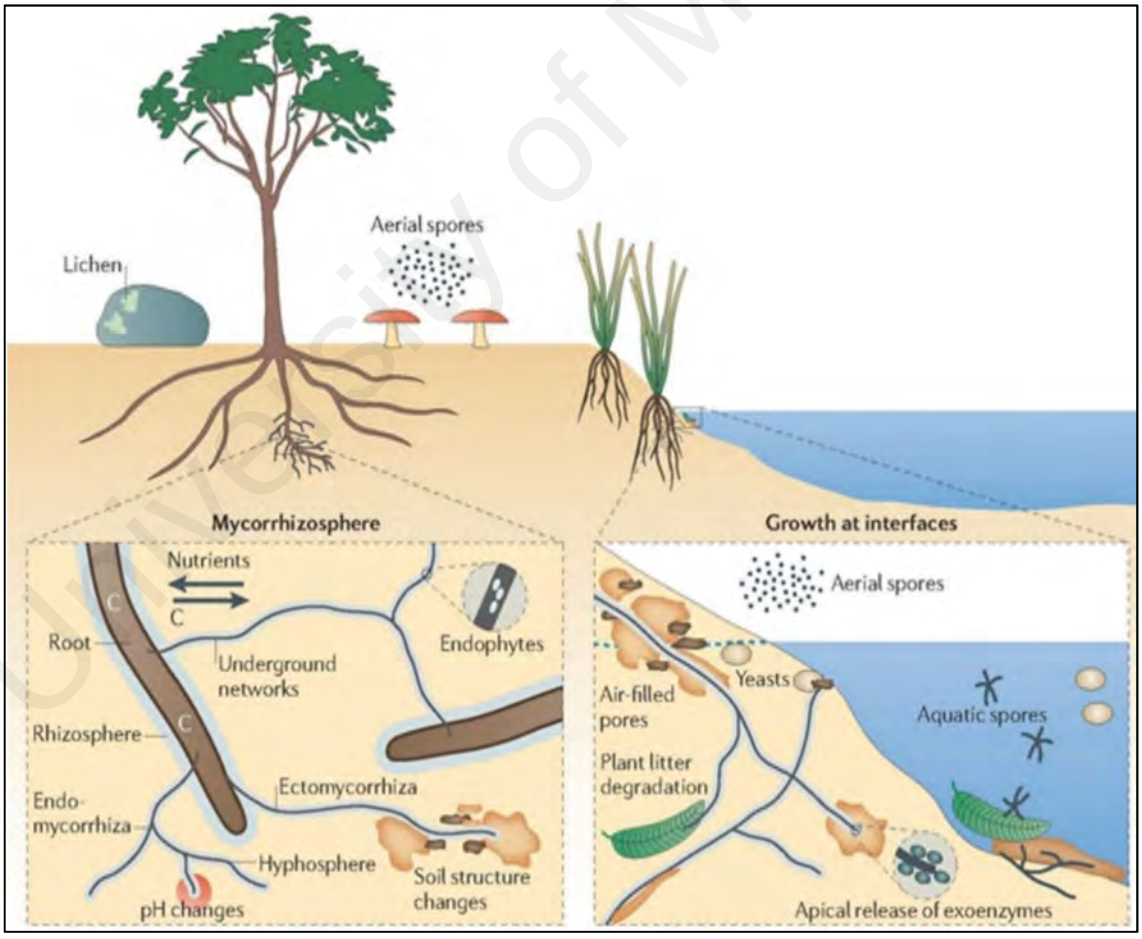


Figure 2.4: Typical habitats of some terrestrial and aquatic fungi (adopted from Harms *et al.*, 2011).

Al-Nasrawi (2012); Ameen *et al.* (2016), and Das and Chandran (2011) have been named also some indigenous fungi from *Ascomycota* group which were isolated from soil, namely, *Penicillium, Aspergillus, Cephalosporium, Fusarium*, and *Cochliobolus* with petroleum deterioration capability at aerated condition. In addition, numbers of non-ligninolytic fungi species from this division and others including *Penicillium*, *Fusarium*, *Aspergillus*, *Mucor, Aureobasidium, Candida, Rhodotorula, Geotrichum, Gliocladium* and many more have been also found to have ability for biodegrading PH in the aquatic aquifers (Okoh, 2006; Xue *et al*., 2015). Moreover, Zafra and Cortes-Espinosa (2015) have been reviewed that plenty *Trichoderma* species with variable efficiency have higher and faster degradation capability of variety aromatic hydrocarbons such as naphthalene which have been found in petroleum hydrocarbons like diesel. For instance, Mishra and Nautiyal (2009), and Hamzah *et al.* (2012) have been discovered that 85% and 20% degradation effectiveness of diesel at concentration of 6000 mg kg^{-1} and 20,000 mg kg^{-1} respectively by *Trichoderma* spp. in soil. Precisely for aromatic hydrocarbons removal as diesel persistance constituent in degradation, Gargouri *et al*. (2015) revealed that two fungi species including *Candida* sp. (97%) and *Trichosporon* sp. (95%) could be used.

There are many reports on white-rot fungi (WRF) such as *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pycnoporus sanguineus*, and *Pleurotus ostreatus* for their potential of degradation of the broad groups of organopollutants by lignin-degrading characteristic (Haritash & Kaushik, 2009). The study of hydrocarbon by white-rot fungi have been initiated by Bumpus *et al.* (1985). Studies done on non-lignolytic and lignolytic fungal isolates from different sources as a petroleum hydrocarbons degrader agent tabulated in Table 2.4. Variance in the degradation rate and efficiency were due to different factors can influence the biodegradation mechanism. Specific medium that have been used are also listed in Table 2.4. While, the isolation and screening approach are similar to each other. Generally, the screening of potential strains have been done by using

the selective media of employing the substrate in recent studies as listed in the table. The enzyme productivity and metabolic fate of organic compounds are very essential in order to achieve and implement the highly efficient bioremediation strategy, thereby, the enzyme production and their chemical molecular pathway to detoxify the organic pollutants captured briefly.

Table 2.4: Listing of study for various fungal strains that can catabolize PH.

Source of Isolation	Fungal Species	Medium	Metabolic Compounds	Findings	References
Oil contaminated soil	*Fusarium sp. F092*	Potato Glucose Medium (PGM)	Aliphatics in crude oil	98% efficient aliphatic fractions (C_{12}-C_{20}) degradation of 1000 mg/L initial concentration under saline condition of synthetic seawater after 60 days.	Hidayat and Tachibana (2012)
	Pycnoporus sanguineus	(PDA), Glucose-yeast-malt-peptone(GYMP)	PAHs	Ligninolytic native fungi degraded phenanthrene, anthracene and pyrene by Laccase enzyme metabolism the end of 20 days with immobilized and free mycelia culture.	Low *et al.*, (2008)
	Aspergillus terreus	(PDA), Mineral Medium (MM), Czapek media	Pyrene, Phenanthrene	The strains were capable for tolerating to high concentration of 2000 mg/L of mix PAHs of pyrene and phenanthrene during 15-day incubation time	Reyes-César *et al.,(*2014)
	Talaromyces spectabilis				
	Fusarium sp.				
	Yarrowia lipolytica	Sabouraud's dextrose agar, Czapek medium, Bushnell-Haas broth	Crude oil	More than 80% of crude oil (1% v/v) were degraded by strains in liquid media after 14 days treatment.	Iheanacho *et al.*, (2014)
	Zygorrhynchus sp.				
	Aspergillus niger	Mineral Salt Medium (MSM)	nC_{17}/pristan, nC_{18}/phytane	The efficient removal of branched alkanes at 1% crude oil after 60 days reported.	Mittal and Singh (2009)
	Trichoderma sp. S019	Mineral Salt broth (MSB)	n-eicosane (alkane)	The degradation ratio of n-alkanes were found 73% and 63% at optimum level of carbon source of glucose and nitrogen source of polypepton respectively after 30-day inoculation.	Hadibarata and Tachibana (2009)
	Polyporus sp. S133	Malt Extract agar (MEA)	Chrysene (PAH)	65% degradation of 10% PAH (4-ring benzene) were obtained in 120 rpm Mineral salt broth (MSB) at 30 days, whilst it was achieved up to 24% at non-agitated condition.	Hadibarata *et al,.* (2009)
	Geomyces pannorum	(MSM)	Diesel oil, Crude oil	Reduction in alkane compound and removal ratio of 77.3 and 43.4% with diesel and crude oil respectively reported for 1% v/v substrate in liquid media over two weeks.	Maddela *et al.*, (2015)

Table 2.4, continued.

Source of Isolation	Fungal Species	Medium	Metabolic Compounds	Findings	References
Oil contaminated soil	*Trichoderma reesei H002*	Malt Extract agar (MEA), Glucose and Yeast Extract broth	Diesel	*Trichoderma* can degrade more than 90% TPHs of diesel oil of 1000 ppm with agitation 100 rpm, pH 6 and in the presence of glucose and yeast extract.	Nazifa *et al.*, (2019)
	Lambertella sp. *Penicillium sp.* *Clonostachys sp.* *Mucor*	Basal Salt Medium (BSM), (MEA), (MEB)	Diesel	Ascomycete was able to promote the degradation of 47.6% of the TPH contamination after two months of inoculation time.	Becarelli S. *et al.*, (2019)
	Aspergillus sp.	(PDB), Mineral Salt Media (MSM)	n-hexadecane (alkane)	The degradation rate increased with an incubation time. *Aspergillus sp*. is able to degrade the targeted substrate more than 86% during 10 days of incubation.	Al-Hawash (2018)
	Penicillium sp.	(MSM), (PDB)	Crude oil	More than 55% degradation percentage was obtained for 1% substrate after 14 days of incubation at room temperature.	
	Cladosporium sp.	Glucose-yeast-malt-peptone(GYMP)	Diesel	Strong degradation potential up to 34% with 1% diesel gained after 5 days of incubation. This strain showed the significant difference in degradation ratio over indigenous bacteria specie of *mycobacterium hyalinum* under same aquatic medium condition.	You-Qing *et al.*, (2008)
	Trichoderma tomentosum	(PDA), (PDB)	Diesel oil, Crude oil, engine oil	Effectual degradation rate of PAHs under extreme condition.	Marchand *et al.*, (2017)
	Fusarium oxysporum				
	Aspergillus spp.	(MSM), (PDA)	Diesel oil, Crude oil, Used engine oil	The highest TPH degradation rate of 63.5% recorded with diesel treatment (The substrate used as a PAH model).	BK and SV (2017)
	Fusarium sp.			The highest TPH removal rate of 69.6% recorded with diesel treatment (The substrate used as a PAH model).	

Table 2.4, continued.

Source of Isolation	Fungal Species	Medium	Metabolic Compounds	Findings	References
Oil contaminated soil	*Aspergillus sp.*	(MSM), (PDB)	Anthracene	Higher conversion of substrate took placed in first 3 days of incubation. Prospect fungi at this study showed fast growing spore formation of fungi in variety range of substrate concentration which can degrade up to 55% of that within 18 days.	Lahkar & Deka (2016)
	Aspergillus sp. *Exophiala sp.* *Trichoderma sp.*	(MEA), Soy-bean flour Suspension	Anthracene	All three isolates at this study showed the complete degradation of anthracene after 42 days of incubation. Transformed anthracene into 9, 10-anthraquinone which is one of the first oxidation products of anthracene described in fungi.	Godoy *et al.,*(2016)
	Aspergillus sp. *Penicillium sp.* *Gleocladium sp.*	(MSM), Bushnell-Haas (BH)	Petrol: Diesel: Kerosene	There was a progressive increase in the amount of CO_2 produced for the first 21 days, after which CO2 production decreased. This is the indication of microbial activity and their role in biodegradation mechanisms.	Dhar *et at.*, (2016)
	Penicillium sp.	(MEA), (MSM)	Motor oil	Complete degradation of aliphatic hydrocarbons (nC_{15}- Nc_{23}) in 1% motor oil obtained over 60 days.	Husaini *et al.,* (2008)
	Aspergillus sp.			The effective aliphatic and aromatic hydrocarbons in 1% motor oil recorded over 60 days.	
Expected Un-contaminated soil	*Aspergillus terreus*	Czapek-Dox agar, Czapek-Dox broth	Naphthalene, Anthracene	Highest enzyme productivity after 10 days with 150 ppm of PAHs were recorded. Degradation efficiency of 98.5% and 91% for naphthalene and anthracene obtained respectively.	Ali *et al.,* (2012)
	Trichoderma sp. S019	(MEA), (MSB)	Phenanthrene	The biodegradation rate conducted up to 72% and 31% for 0.1 and 1.0 Mm of 3-ring PAH at liquid medium after 30 days.	Hadibarata *et al.,* (2007)

Table 2.4, continued.

Source of Isolation	Fungal Species	Medium	Metabolic Compounds	Findings	References
Mangrove forest soil and tarball samples	*Aspergillus niger*	(PDA)	kerosene	Almost 20% removal of contamination (20% v/v) achieved after 10 days inoculation.	Lotfinasabasl *et al*., (2012)
	Rhizopus sp.				
Mangrove sediments	*Cladosporium sphaerospermum*	(MSM), (PDA)	Diesel	It can degrade diesel up to 34% after 5 days treatment.	Ameen *et al*., (2016)
	Eupenicillium hirayamae			Showed the highest catalase enzyme production up to 14.0±0.99 U/ml, and maximum biomass of 43.4% and maximum CO_2 liberation of 72% after 20 days.	
Refinery wastewater	*Candida tropicaalis*	(MM)	Hexadecane (alkane)	Effective degradation ratio of TPH over 95% during 20 days.	Gargouri *et al.*, (2015)
	Trichosporon asahii				
	Trichoderma virens	(PDA), (MSM)	Crude oil	40% removal of TPH preferably C20-C40 chained fractions reported with 1% of substrate after 9 days.	Hamzah *et al.*, (2012)
	Rhodotorula mucilaginosa	Yeast Extract (YM), (MEA), (MEB)	Lipid production	Glucose and peptone were identified as best carbon and nitrogen source for high biomass induction.	Prabhu *et al.*, (2019)
Sludge contaminated soil	*Aspergillus flavus*	(PDA), (MSM)	Diesel	Exhibited higher biodegradation efficiency with 1% v/v diesel in oil agar media after 10 days	Chaudhry *et al.*, (2012)
	Aspergillus niger				
	Alternaria sp.				
	Penicillium sp.				
	Fusarium sp.				

2.3.2 Biodegradation Mechanism of Fungi toward PH

Fungal enzyme's activities and its production have been studied extensively in the respond to variety pollutant compounds and condition (Saratale *et al.,* 2007). Incorporating relatively non-specific enzymes for attacking and oxidation of organopollutants such as petroleum hydrocarbons in the ecosystem is the unique feature of fungi. These enzymes probably participate in the range of metabolic reactions for oxidation and mineralization of the pollutants substrates with random structure, e.g. lignin and its derivatives- which is unavailable for bacteria- and gain energy requirement for their biomass growth (You-Qing *et al.,* 2008). Fungi are incorporating three kinds of enzymes namely, oxygenase, dehydrogenase, and lignolytic enzymes for hydrocarbon degradation which basically are secreted either extracellularly or intracellularly (Harms *et al.,* 2011).

Ligninolytic enzymes of fungal are lignin peroxidase (LiP), laccase (Lac), manganese peroxidase (MnP), and catalase (Kota *et al.*, 2014). They are released extracellularly and destabilize carbon bonds in a molecule by oxidation of radicals to carbon dioxide (Haritash & Kaushik, 2009). The use of ligninolytic fungi, i.e. white Rot fungi (WRF) with particular extracellular lignin degrading enzymes has been well studied in mycoremediation strategies (Koyani, 2011; Low *et al.*, 2008; Reyes-César *et al.*, 2014). White Rot fungi can enhance the bioavailability of broad range of organic compounds for removing with their serious of various enzymes, however, the growth rate of these species are very slow and stand in needs of rich oxygen condition (Wu *et al.*, 2010). Meanwhile, cytochrome P-450, monooxygenase, lipases, and epoxide hydrolases are being secreted by non-ligninolytic fungi. They are classified as either extracellular or intracellular enzymes of fungal species (Kadri *et al.,* 2017). These class of non-white-rot fungi can evidently succeed in dealing with a quite low oxygen condition (Wu *et al.,* 2010).

Nevertheless, it is reported that PH can induce the secretion of extracellular Lac enzyme in some species of non-ligninolytic fungi as well (Kadri *et al.,* 2017). Figure 2.5 illustrates simple degradation process of hydrocarbons by non-ligninolytic fungi which initially includes extracellular oxidation and intracellular analysis under cytochrome P-450, monooxigenase, and epoxide hydrolase enzymes. Products arising from extracellular contamination oxidation may undergo to further intracellular metabolism or may subject for origination of bound residues and CO_2. Final metabolites derived from initial attack of intracellular enzymes may be discharged as the metabolite excretion or influenced by extracellular enzymes activities to form bound residues. In addition, they may be secreted and stored as conjugates in the nature which are inactive and persistent compounds (Marco-Urrea *et al.*, 2015).

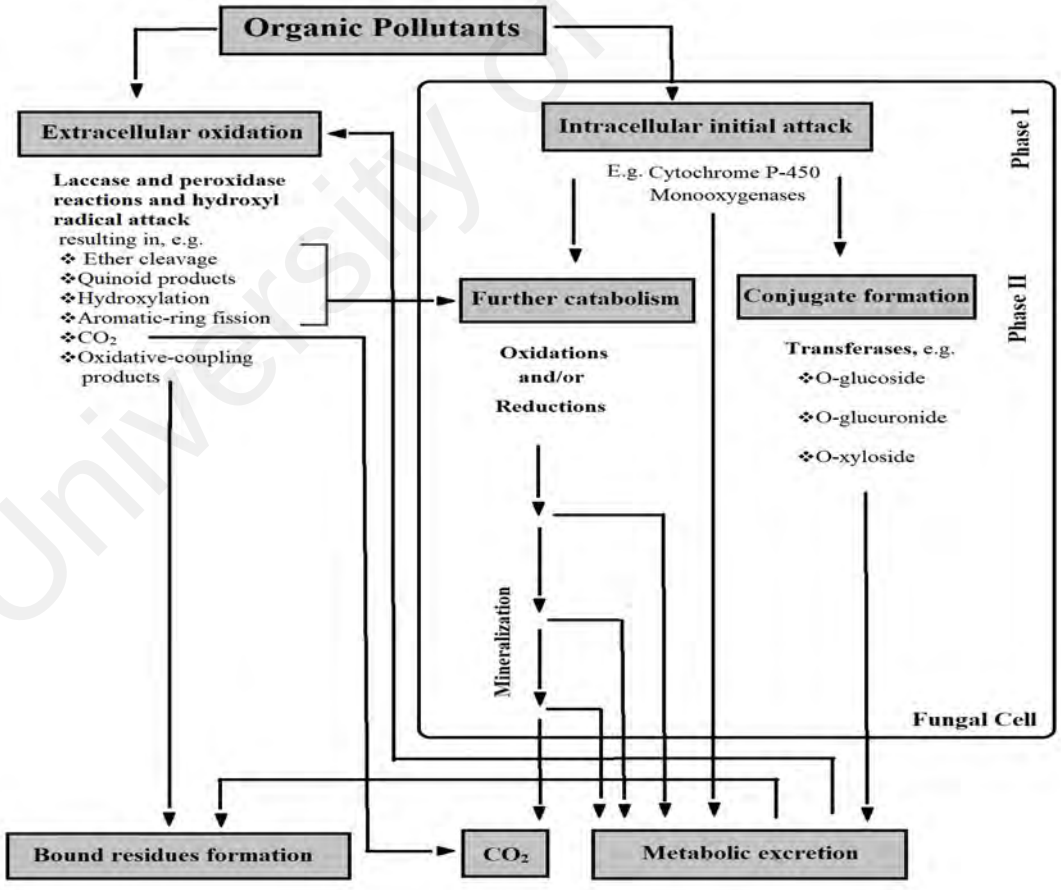


Figure 2.5: Simplified schematic process of non-ligninolytic fungi to degrade organic pollutants (adapted from Marco-Urrea *et al*., 2015; Kues, 2015).

Since the petroleum hydrocarbons own the mix variety structural constituents and toxicity; it is expected to have different functional enzymatic mechanism with various components (Okoh, 2006). Ameen *et al.* (2016) have been determined tremendous lignolytic enzymes activity (LiP, MnP, Lac, and catalase) in diesel contaminated medium treated by *Aspergillus, Cladosporium*, *Eupenicillium, and Paecilomyces* species of fungi. Moreover, Zafra and Cortes-Espinosa (2015) has been suggested that along with related enzymes of laccases and peroxidases, particular subsidiary kinds of cytochrome P-450 oxidases, e.g. 1-2, 2-3 extra-diol dioxygenases can release by *Trichoderma* species to be involved in PAHs degradation.

In the case of alkane's substrate oxidation, depending on the length of carbon chain, there are three different enzymes system. Principally, alkanes in the range of carbon numbers from C_1 to C_4 will induce the production of methane monooxygenase enzyme, C_5-C_{16} will be oxidized by non-heme membrane monooxygenase and P-450, whilst C_{17+} will catalysed by unknown system (Van Beilen & Funhoff, 2007). Several fungi isolates have been investigated for the enzymatic metabolisms on degradation of n-alkanes. The enzyme productivity and accumulation in *Aspergillus* sp. has been documented in the study by Al-Hawash *et al.* (2018), where the generation of alkane hydroxylase, alcohol dehydrogenase, and cytochrome P-450 enzymes reported in degradation of n-hexadecane as a representative model of alkane substrate.

Aromatic hydrocarbons are widely oxidize by two metabolisms of cytochrome P-450 and ligninolytic extracellular enzymes, i.e. lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (phenol oxidase, Lac) (Kadri *et al*., 2017) which can produce by different fungal strains (Gupte *et al.*, 2016; Peng *et al.,* 2008). *Aspergillus* sp., *Fusarium* sp. and *Trichocladium* sp. have been reported for secreting ligninolytic enzyme

to degrade low-molecular weight of aromatics under limited oxygen condition (Hassanshahian & Cappello, 2013).

2.3.2.1 Biochemical Degradation Pathway of PH biodegradation

The use of fungi to bio-remediate PH has been studied; and based on the study, the basic principle of biodegradation process by fungi has been elaborated. In biodegradation process, fungi take energy and detoxify the PH as sole carbon source into their less hazardous/non-hazardous substances and cell biomass with less input of energy and time (Haritash & Kaushik, 2009). Generally, three different segments of fungi are responsible for this degradation, i.e., absorbance of PH by microbial surface, PH degradation through microbial cell membrane and ultimately oxidation of these compounds into simpler molecular by microbial cell (Xue *et al*., 2015). All these chemical mechanisms materialize by fungal extracellular and intracellular enzymes (Kadri *et al*., 2017) which was explained in 2.3.2 section.

This chemical procedure can take place under either phototrophic, aerobic or anaerobic condition (Varjani, 2017). The basic potential pathways of hydrocarbons degradation by fungi is illustrated in Figure 2.6 (Das & Chandran, 2011; Varjani & Upasani, 2017), which is starting with fratctionizing pollution into less hazardous compound under three mentioned enviroinmental condition. Many studies have been solely focused on the pathway of mineralization of hydrocarbons under presence of oxygen. However, there are numbers of particular microorganisms (mostly bacteria) which have been documented in recent literatures for biodegradation route of PH under anaerobic condition (Meckenstock *et al*., 2016; Widdel & Rabus, 2001). However, this degradation pathway by fungi usually takes place under existence of oxygen. Oxidation, hydroxylation, dehydrogenation, and mineralization are the commonly reactions participating in both aerobic and anaerobic

metabolic pathways of PH pollutants biodegradation by fungal microbes (Varjani & Upasani, 2017).

Binding to particulate matter of petroleum hydrocarbons make them difficult to degrade by microorganisms. However, degradation rates are vary depending on capability of microbial attack which has found for crude oil- has relatively same composition with diesel oil- as following rank, i.e., linear alkanes > branched alkanes > small and simple aromatics > polycyclic aromatics (Howard *et al*., 2005). Although, even some higher molecular weight such as polycyclic aromatic hydrocarbons (PAHs) might not be degraded forever (Das & Chandran, 2011; Varjani & Upasani, 2017) or the rate of degradation of these substances can reduce, if fungi involve in the competitive inhibition of the more readily compounds (Abdel-Shafy & Mansour, 2016). Meanwhile, majority of organic pollutants such as PH completely are degraded in faster aerobic condition (Das & Chandran, 2011). Oxidation is the initial intracellular attack of PH by microorganisms which activates the PH molecules for catalysing by oxygenase and peroxidase enzymes. Latter, pre-intermediate compounds through several peripheral degradation pathway transform into either H_2O or CO_2 via respiration action or cell biomass via central aerobic intermediary metabolism of Krebs cycle (TCA) or via anaerobic biosynthesis pathway (Das & Chandran, 2011).

A part from that, it was stated that different fungal species employ independent specific metabolic pathway for degradation of targeted pollutants (Zafra & Cortes-Espinosa, 2015).

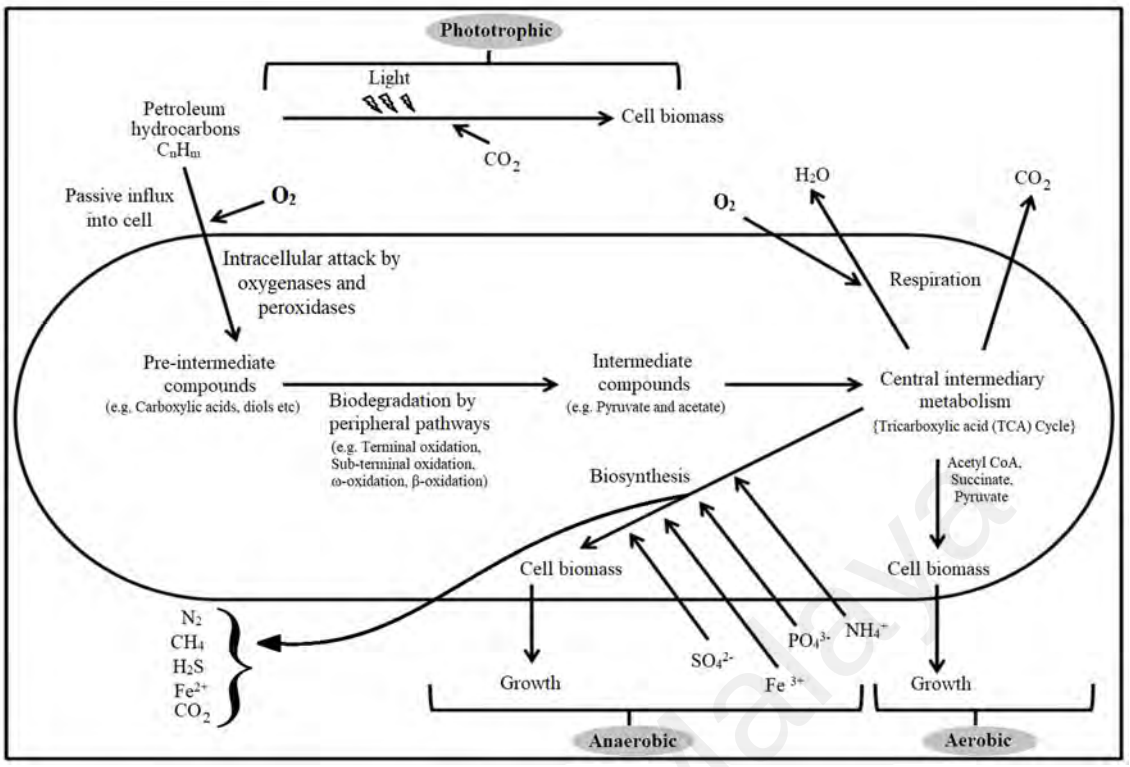


Figure 2.6: Feature for principle of degradation pathway of PH by microorganisms under different conditions (adapted from Das & Chandran, 2011; Logeshwaran *et al*., 2018).

i. Aerobic Biodegradation Pathway:

At this process, petroleum hydrocarbons (e.g., alkanes, cycloalkanes, aromatics) undergo the various metabolic ways due to the different molecular structures. The key element of this process is oxygen atom. Addition of oxygen is important for activation of aliphatic and aromatic hydrocarbons in petroleum fuel to transform into alkanol and phenol respectively (Hassanshahian & Cappello, 2013).

Since organopollutants with high quantity of carbon and hydrogen contents and low oxygen atom classified as hydrophobic contaminants this pre-chemical activation process is the initial phase of activation of hydrocarbon's molecules to be solved more in water and get the site ready for well- organized upcoming reactions (Leitao, 2009; Shukor, 2013).

Alkanes and cycloalkanes are the most readily fractions in the bio-detoxification process by fungi. Different enzymes catabolism are employ for the detoxification of these groups of hydrocarbons in the diesel. The aerobic degradation pathway mechanism of alkanes by fungal enzymes involves the similar central intermediates and end products as bacterial enzymes techniques (Shukor, 2013; Sierra-Garcia & de Oliveira, 2013). The fundamental biological degradation of these saturated hydrocarbons are identical which can be systemized in the frame of terminal and sub-terminal oxidation, alkyl groups (alcohol and aldehyde) hydroperoxides, ω-oxidation, β-oxidation, and deterioration of cyclohexane (Figure 2.7) (Varjani, 2017; Xue *et al.*, 2015). Intermediate products of fatty acids produce in the first phase of degradation of alkanes by monooxygenases through alcohol and aldehydes under aerobic condition (Okoh, 2006). Then the acetyl-CoA derived from these acids through β-oxidation in Tricarboxylic acid Cycle (TCA) could be composed into carbon dioxide (CO_2) and chemical energy.

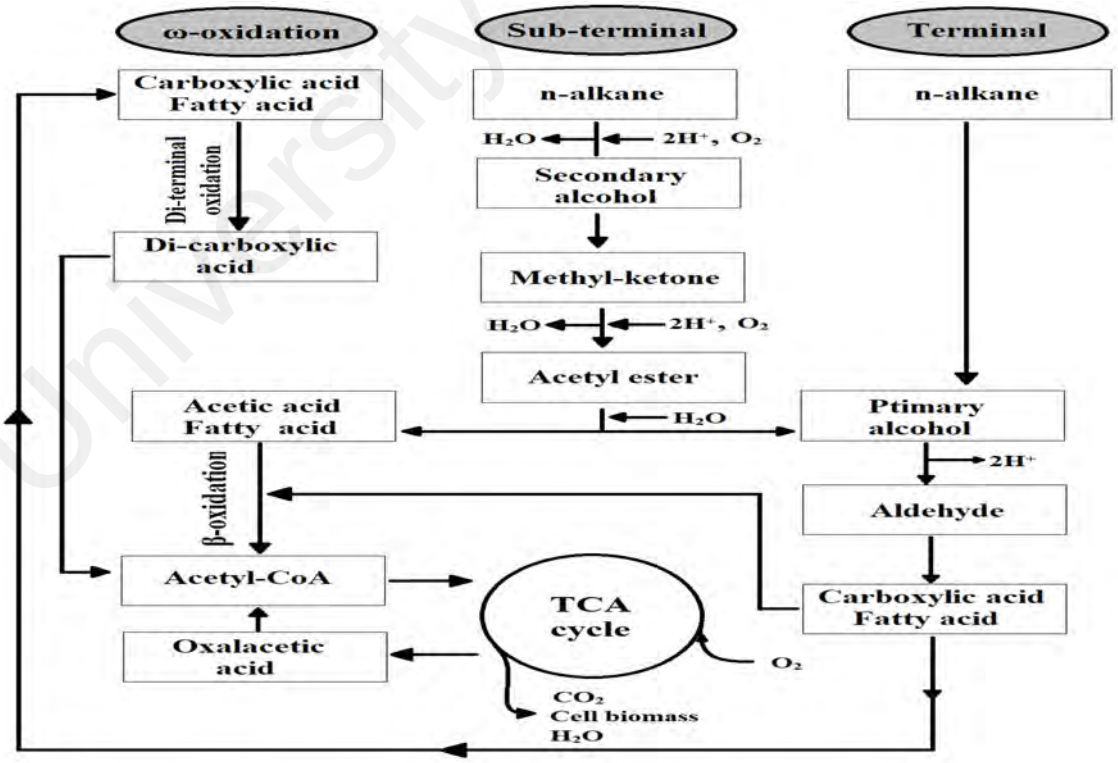


Figure 2.7: Representation of principal enzymatic pathways of n-alkane aerobic degradation by fungi (adapted from Ma, 1998; Okoh, 2006; Varjani, 2017).

However, in some cases, fatty acids can accumulate in the ecosystem after excreted by microorganisms' cells (Hassanshahian & Cappello, 2013). In the case of cycloalkanes, they are subjected to transform into a corresponding cyclic alcohol through oxidase reaction, then after losing the hydrogen atom converted into ketone. Final ring fission by oxygenase system result in lactone hydrolase which is not usually fully completed with the same species of microorganisms (Okoh, 2006).

Aromatic hydrocarbons are more recalcitrant in the ecosystem and to biological degradation process by microorganisms. Besides, their degradability rates depend on phylogenetic spectrum of microorganisms and chemical structural composition of aromatic molecules (Gupte *et al*., 2016). Generally, with almost up to 3- or 4- benzene rings aromatic compounds in the diesel fuel, there are three fundamental aerobic pathways including of dioxygenase, monooxygenase and unspecified oxidation which are the cleavage of carbon rings for formation of linear acids. Complete mineralization of the aromatic hydrocarbons through dioxygenase mostly is performed by bacteria (Lease, 2006). During this process, one benzene ring of compound is attacked and oxidized into the final products of biomass and carbon dioxide derived from main intermediates of pyruvate group (Ma, 1998). Establishment on only up to four carbon rings compounds is the limitation of this route (Hassanshahian & Cappello, 2013).

Monooxygenase pathway is usually up taken by fungi and yeasts. During this chemical oxidation one or two oxygen atom are introduced to available compounds via monooxygenases enzymes (Laese, 2006). The highly reactive epoxides (arene oxide) is the main intermediates in this activity which may further yield to cis/trans-dihydrodiols or phenol that exposed to non-enzymatic reaction leading to inactive products stored in nature (Gupte *et al*., 2016).

Unspecific oxidation sequence have been stablished by ligninolytic fungi mainly white-rot fungi. Lignin structure undergoes by extracellular peroxidases and phenol oxidase enzymes destroy the phenolic molecular structure of quinones. Epoxides and dihydrodiols have high toxicity potential more than their parent compounds, unlike, the end product of oxygenase reaction by ligninolytic fungi is quinines which is less toxic than the initial compounds, therefore, degradation of PAHs is more advantageous by exploiting white-rot fungi (Ghosal *et al.*, 2016). The main principal of microbial metabolisms pathway of aromatic hydrocarbons illustrates in Figure 2.8 by ligninolytic and non-ligninolytic fungal enzymes in comparison with bacterial dioxygenases. Aromatic hydrocarbons may also be attacked and ring fission by these metabolisms of fungi and transformed into compounds with a carbon ring number (s) less than that in the initial composition and detoxified products (Leitao, 2009).

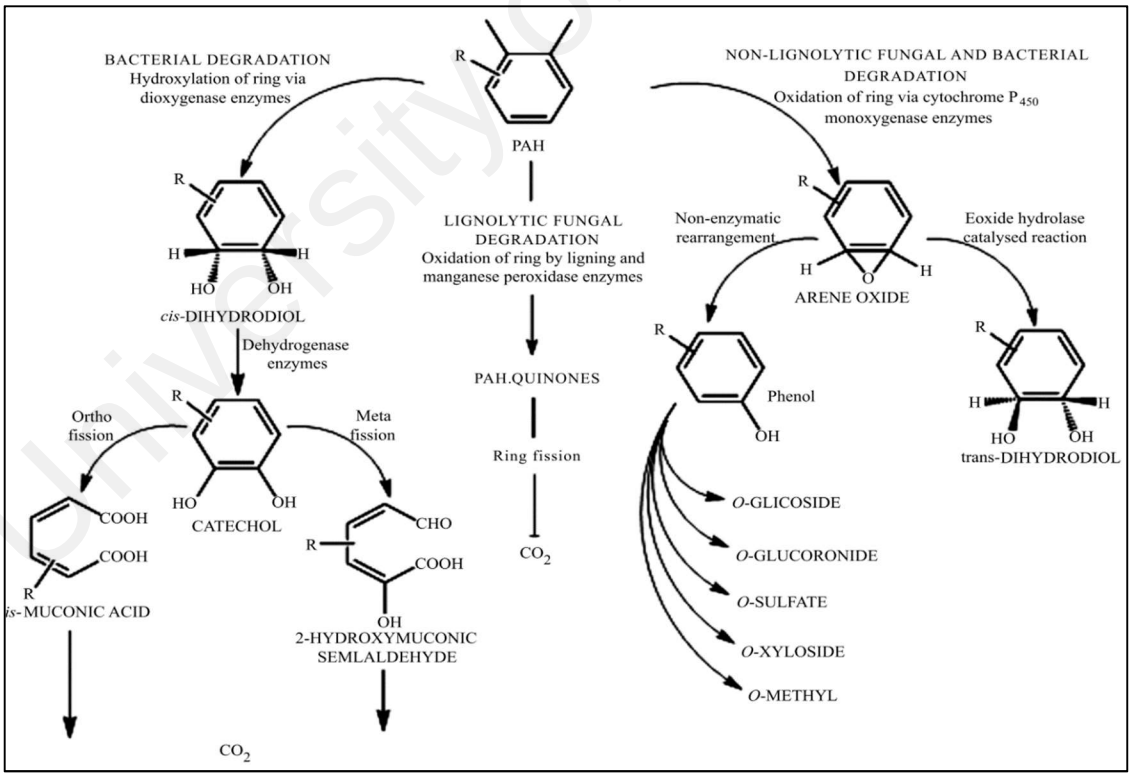


Figure 2.8: Fundamental pathway for aromatic hydrocarbons degradation by microbial enzymes (adopted from Gupte *et al*., 2016).

High energy generation on the duration of aerobic pathway which is being utilized by microorganisms to synthesize and grow, makes it favourable for ending with better quality effluents. However, the demand for high-level of oxygen consumption is the one of the main disadvantage of this degradation process (Gupta *et al.,* 2015).

ii. Anaerobic Biodegradation Pathway:

Recently, many studies have been focused on only the mineralization of hydrocarbons under presence of oxygen. However, there are numbers of literatures which reviewed and reported the biodegradation of PH in a state of anaerobic circumstances. In this chemical metabolism comparing with aerobic pathway, different electron acceptors including nitrate, ferrous iron, sulphate, and/or manganese ions are used for degradation route of PH (Varjani, 2017).

Energy requirement for this process is less than that on the aerobic condition; this is due to the absence of aeration as well as final by-product of methane which is beneficial for biofuel-energy production and temperature control at anaerobic processes. On the other hand, low biomass production in the long period of time and the microbial sensitivity to the temperature alteration are the major drawbacks of degradation under lack of oxygen (Gupta *et al.,* 2015).

2.3.3 Important Factors for Biodegradation

The approach and grade of biodegradation depends on numbers of physical, chemical and biological factors which may affect successfulness of implementation of biodegradation process in aquatic ambient and may differ from site to site (Haritash & Kaushik, 2009). Physical or abiotic factors includes physical and chemical composition and characteristics of substrate, concentration of accumulation, oxygen, pH, temperature, bioavailability, inorganic nutrient content, salinity, pressure. Meanwhile, Biological or biotic factors

consists of microbial bioavailability, presence of proper microorganisms to detoxify target substances, enzyme activities (Francis-Obika, 2004; Singh & Chandra, 2014).

2.3.3.1 Physio-chemical Factors

External parameter of temperature plays a significant role especially on practical in situ biodegradation. Temperature alteration can effect on molecular chemistry of contaminants as well as physiology and population of microbial community (Okoh, 2006). Increase and decrease in temperature will affect the viscosity (pollutants distribution) and breaking rates of chemical fractions and as a consequence chemical solubility in water column may influence by fluctuation of temperature (Atlas, 1981; Margesin & Schinner, 2001), final will impress the competence of the biodegradation process. However, augmentation of natural attenuation in some extent can terminate to stretch the biodegradation process. Therefore, Das and Chandran (2011) studied the optimum temperature requirements for maximum degradation rate in aquatic environment separately ranging from 15-20 ºC for marine environment, and 20-30 ºC for fresh water. Generally, biodegradation rate decreases with dropping the temperature (Okoh, 2006).

One of the other limiting factors for biodegradation is nutrient content specifically nitrogen, phosphorous and in some cases iron. Several studies have been investigated that additional or absence of nutrient supply has much more effect in fresh water wetlands and marine environment due to poor availability of these compounds (Das & Chandran, 2011). Unlike, the volume of these substances for enhancing the biodegradability of pollutants is fluctuating due to the constant effluent discharges into fluent fresh water reservoirs (Okoh, 2006). As a further matter, the biodegradation rate of hydrocarbons can favourable with introduction of carbon source of glucose and nitrogen source of sodium nitrate (2 g/L) to medium (Ali *et al*., 2012; Leitao, 2009). Moreover, agro-industrial

wastes, e.g. wheat straw, corn stalks can also support the biomass production of exogenous microorganisms to survive and degrade xenobiotic in the polluted site by serving them as the carbon, nitrogen and phosphorus (Cortés-Espinosa & Absalón, 2013).

Oxygen as an electron acceptor in the major aerobic enzymatic reactions of oxygenises engages a critical role to extend the biodegradation rate (Varjani & Upasani, 2017). The significant element of both mono- and dioxygenase enzymes metabolic pathway of aromatic's initial oxidation is oxygen atom. For this reason, external serving O_2 in terms of direct aeriation, tilling, drainage or chemical supplements which generate oxygen free radicals suggested for in-situ aquifers bio-mitigation enhancement of BTEX compounds and PAHs (Ghosal *et al.,* 2016). 52 % detoxification of 200 mgL^{-1} phenanthrene was conducted with 20 % of oxygen provision, on the other hand, degradation trend reached only 13 % under relatively oxygen shortage condition (Leitao, 2009).

Concentration, composition, and material properties of pollutants also control the deterioration rate. Regularly, with increasing of volume, weight and complexity of chemical structures, biodegradation will decrease. In this sense, degradation scale of n-alkane hydrocarbons are notably higher than branched and cyclic ones with higher number of carbon (Xue *et al.,* 2015), which is commenced to breakdown preferably with carbon chain number ranging from C_{10}-C_{25} to C_{25}-C_{40} and eventually complex alkanes (Koshlaf & Ball, 2016). Ali *et al.* (2012) reported degradation rate efficiency of PAH with lower carbon rings by Aspergillus relatively total naphthalene (2-rings) removal of 98.5% after four weeks of treatment observed in contrasting to anthracene (3-rings) mineralization of 91% for the same duration. Final detoxification and eliminating of pollutants in the given ecosystem is the main aim of bioremediation techniques. Thus, monitoring the biodegradation pathway under microbial enzymes activities and considering efficient operation system are crucial in order to be aware of hazardous dead

end-products generation from these catabolic routes before and after treatment (Ghosal *et al.,* 2016). General talking, microorganisms can tolerate and adapt for growth or degradation of hazardous level of contamination if the concentration of pollutants is less than their toxicity threshold (Santos *et al.*, 2011).

Bioavailability of hydrocarbons is the other limiting factor can influence the biodegradation rate of these compounds by microbial cells. As molecular mass of hydrocarbons increases, the solubility in water will decrease terminating into less availability for microbial cell attack (Koshlaf & Ball, 2016). Enrichment the petroleum oil impacted site and medium by bio- and non-ionic synthetic Surfactants will also enhance the biodegradation process by increasing hydrocarbons bio-accessibility and microbial cell contacts with substrate (Varjani & Upasani, 2017). The superiority incorporating surfactant in biodegradation improvement may also refer back to their ability to enhancing the petroleum compounds mobility and shrinking the interactional tension through cell membrane permeability adjustment (Hadibarata & Tachibana, 2009). Bio-surfactants have been known for better ecosystem's compatibility and high selectivity under extreme environmental condition as well as low toxicity than synthetic ones. For instance, Rhamnolipid bio-surfactant produced by *Pseudomonas aeruginosa* could amplify the biodegradation rate of n-alkanes from 5% (without surfactant) to 20% at 90-hours laboratory experiment (Rashad, 2001).

Fungi remark with pH sensitivity rate of 5.0 to 7.8 for essential petroleum oil degradation, hence, the pH value of the polluted area was considerably above or below this range, then the degradation efficiency will decline(Das & Chandran, 2011; Salb, 2001). Moreover, it was suggested in the study by Ali *et al*. (2012) maximum level of extracellular enzyme productivity for degradation application of low weight PAH compound was estimated in the acidic range of pH. Therefore, the pH values are in the

below or above optimum level has detrimental effects on growth rate and enzyme productivity of microorganisms, as Maddela *et al.* (2015) at his study showed the sensitivity of growth rate of isolate to changing the pH condition from ideal level of 5.0 in degradation of diesel and crude oil.

Salinity and pressure point are becoming limiting factors for degradation of pollution in saline resorts such as deep sea and marines. Principally, the reduction of degradation rate for hydrocarbons was reported as the volume of dissolved salt in water column becomes less or higher than optimum level (Rodricks, 2001). Moreover, Martins and Peixoto (2012) reviewed that the high salt volume in the environment can cause limitation on the carbon and oxygen sources accessibility and biodegrader microbial inhabitants and performances. In this sense, not only no biomass growth, but also negligible removal of hexadecane were discovered at salt concentration of above 250 g/L in medium, wherever 50 % of removal was obtained at 33 g/L salinity level for same duration time (Martin & Peixoto, 2012). Furthermore, it is stated that there is linear constant relation of salinity and hydrocarbon toxicity in water bodies which in turn it is based on the numbers of hydrocarbon molecules that can dissolve in water column decreased (Fingas, 2016).

2.3.3.2 Biological Factors

Biological factors which can affect the efficiency of biodegradation process have been studied extensively. It is obvious, availability and number of microorganism community which are capable of removing organic pollutants is vital in polluted site. Employing appropriate microorganisms in order to efficient bioremediation approach of polluted site under favourable situation is one of the crucial factor (Singh & Chandra, 2014). The knowledge of suitable microbial population would assist the prior adaptation and augmentation action as two main factors for governing the successful degradation strategy (Rodricks, 2001).

There are several biological techniques which can enhance the PH bioremediation by microorganisms in water medium. Biostimulation, bioaugmentation, phytoremediation, surfactants, cooperated degradation and bio-reactors might employ either together or alone to amplify the biodegradation rate of substrates by microbial culture (Wick *et al*., 2011). For instance, the use of tween 80 as a surfactant folded up two times the degradation ratio of 10% chrysene (4-ring PAH) up to 49% by Polyporus sp. S133 in liquid media after 30 days of treatment (Hadibarata *et al.*, 2009). In another study by You-Qing *et al*. (2008) for cooperated degradation factor, employing two strains of *Mycobacterium hyalinum* (bacteria) and *Cladosporium* (fungi) at the same time in liquid medium suggested. Up to 99 % removal of total diesel have achieved during five days of treatment ; whereby this rate achieved only about 34% by fungous isolate itself and nearly zero percent with bacteria strain.

To conclude, understanding whole internal and external physio-biological elements are fundamental for well- design and successful bioremediation application approaches and must be determined before implementation strategies (Santos *et al*., 2011).

CHAPTER 3: METHODOLOGY

Study techniques were started with isolation and screening of different soil fungi to ending by extended biodegradation study are given in the Figure 3.1.

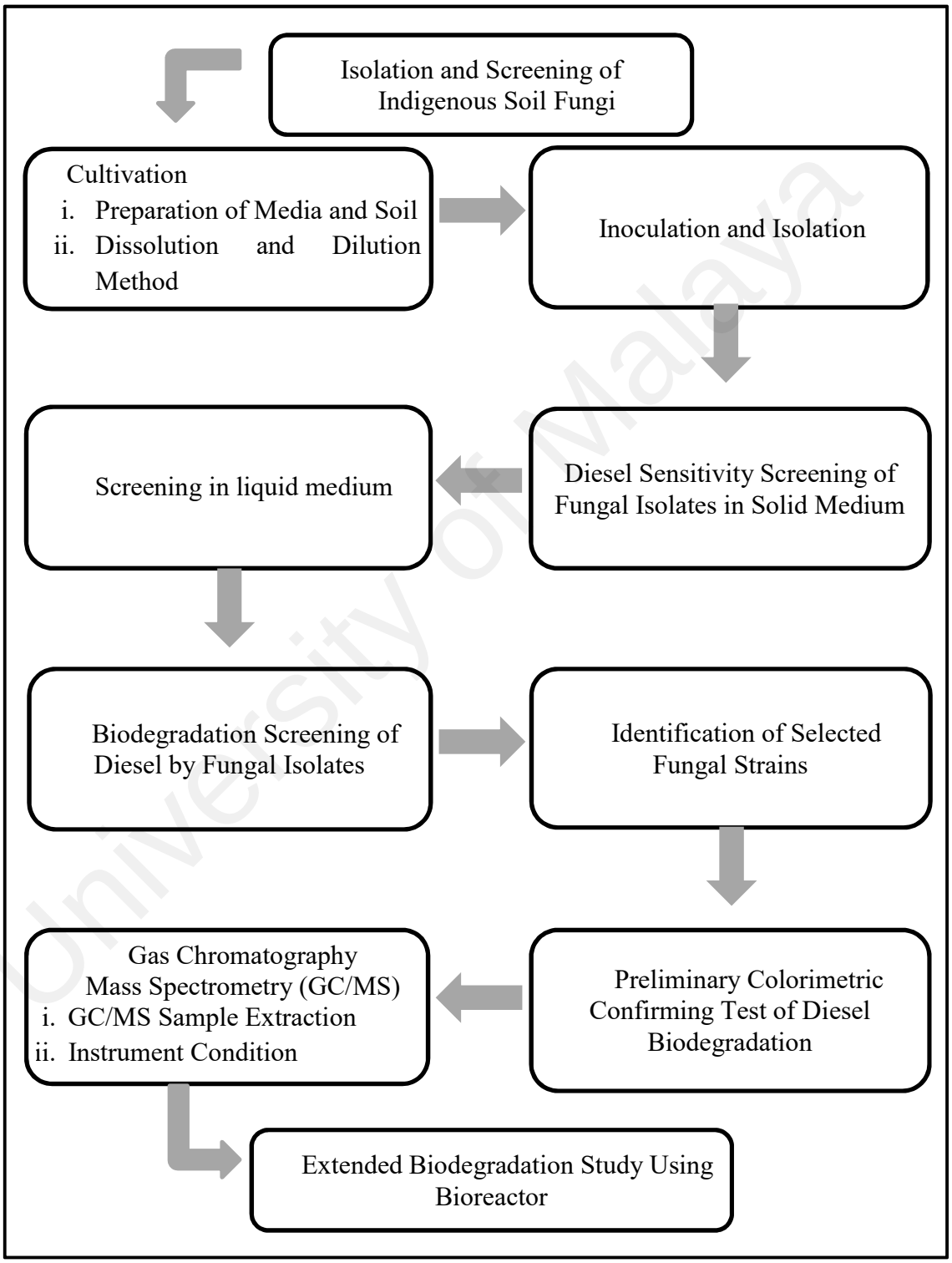


Figure 3.1: Schematic diagram for laboratory work.

3.1 Isolation and Screening of Soil Fungi

Soil samples (500 g each area) for biodegrading fungi isolation experiment of this study were taken (surface top soil 1- 5 cm depth) from three different locations in Kuala Lumpur with variety structure and specification. Each sample were obtained from 3-4 random locations per plot.

a) Estimated natural soil: was collected from garden soil in 3.130652 latitude and 101.657587 longitude at University of Malaya's Rimba Ilmu garden.
b) Runoff soil: the runoff water compile and release to sewerage system may encountered risk of contamination located at 3.133965 LAT and 101.682852 LONG.
c) Petroleum contaminated area soil: from recently petrol station zone with newly fresh soil alteration on top with 3.133637 LAT and 101.683416 LONG.

3.1.1 Cultivation

i. Preparation

Apparatus

a) Sieve (2 mm mesh)
b) pH meter (Sartorius PB-10)
c) test tube

Materials:

a) Soil
b) Distilled water

Procedure:

For culturing preparation, all big debris and particles were removed, then samples were sieved (Kota *et al*., 2014). pH of all samples were estimated on suspension on distilled water of each source of soil on test tube which were indicating the same pH result about

pH ≈ 6.36- 6.57. Soil then were air dried at room temperature and preserved at 4 ºC for further use (Maddela *et al*., 2015; Covino *et al.*, 2015; AI-Jawhari, 2014).

ii. Dissolution and Dilution Method

Apparatus

a) Vortex mixer (VM-300)

b) Pipette

c) Pipette tips

d) Measuring Cylinder (10 ml)

e) Sterile test tube (15 ml)

Materials:

a) Distilled water

b) Soil

Procedure:

Fungal isolation and individual isolated strains culture based on methodology was used by Ferrari *et al.* (2011) and Godoy *et al.* (2016) with some amendments. In sterile plastic tube, the suspension of 1 g of each soil sample and 10 ml distilled water were vortexed for about 2 min and then large particles were allowed to settle for further 2 min (Ferrari *et al*., 2011; Lahkar & Deka, 2016). Before spreading on plates, serial dilution up to a concentration of 10^3 were prepared (Gupta, 2012; Vanishree *et al.*, 2014). The use of high dilution factor assist to start with single and separate spores as well as makes a favour for isolation of fungous type (Nevalainen *et al.,* 2014).

3.1.2 Inoculation and Isolation

Apparatus

a) Pipette (10 µl, 100 µl, 1 ml)

b) Pipette tips

c) Sterilize petri dishes (15× 9 mm)

d) Auto clave

e) Laminar hood

f) Bunsen burner

g) Bent glass rod (spreader)

h) Laboratory parafilm

i) Cork borer (1 cm^2 cutter), forceps and needle

Materials

a) Manufacture ready-made Potato Dextrose Agar (PDA): Typical composition (g/L): potato in fusion 4.0 (fusion from 200g potatoes), D (+) glucose 20.0 g, Agar 15.0 g, pH: 5.6 ± 0.2 at 25 ^oC, and was sterilized at 121 ^oC for 15 min.

b) Chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$) (For reducing the risk of bacterial contamination and getting infection by other microorganisms)

c) Diesel: was purchased from local petrol station in Kuala Lumpur and stored at room temperature in dark place.

d) Acetone (C_3H_6O)

e) Distilled water

Procedure:

Step 1: Sterilized petri dishes were covered by 20 ml of autoclaved PDA supplemented with 1000 µl/L of chloramphenicol.

Step 2: Before solidification of PDA, while it was warm (below temperature of acetone boiling point: 56 ^{o} C), diesel fuel was dissolved in pure acetone in 1:1 ratio for the final concentration of 1% (v/v); and was incorporated to the petri dishes. According to report by Marchand *et al.* (2017), cultivation in selected media (oil-coated medium) would be the best way for isolation of microorganisms, which are able to metabolise some compounds of targeted substrate efficiently. The concentration of diesel in medium was

prepared by dilution of diesel substrate in certain amount of liquid and calculated by following formula:

$$\left(\frac{X\ mL}{X\ mL+M\ mL}\right) \times 100\% \quad \text{..... Equation 1}$$

Where the X is concentration of substrate and M is the volume of medium.

Step 3: By using sterile pipette, 0.1 ml of the diluted soil suspension up to 10^3 X were transferred into the petri dishes. Due to homogenizing texture, Spread Plate techniques (Kota *et al*., 2014; Gupta *et al*., 2012) by using flamed-sterilized spreader was used in order to proper distribution. All plates were wrapped with parafilm and incubated at room temperature for 7 days with daily observation. Control plates were preserved only PDA and PDA with diesel in the same condition. All plates were cultured in triplicate with each soil sample.

After completion of cultivating period, by using flamed-sterilized cutter, forceps and needle, a peripheral disk (approximately 1 cm^2) of each distinct morphotype of fungal mycelia were cut from the agar, and transferred into the fresh PDA plates, which was amended with 1% (v/v) diesel at same condition as previous experiment (Chaudhry *et al*., 2012; Godoy *et al*., 2016; Gupta *et al*., 2012). This process was repeated five times aseptically and sub-cultured on same culture media, until pure fungal culture was acquired. Pure culture was obtained from colony morphological feature which is unique for each isolate. All obtained cultures then were preserved in PDA plates at 4 ºC temperature for longer keep and sub-cultured every three months.

3.1.3 Diesel Sensitivity Screening

i. Solid Media

Apparatus

a) Pipette (10 µl, 100 µl, 1 ml)

b) Pipette tips

c) Olympus light macroscopy (S751)

d) Vernier calliper measurement

e) Laboratory parafilm

f) Cork borer (1 cm^2 cutter), forceps and needle

g) Bunsen burner

h) Laminar hood

i) Sterilize petri dishes (15× 9 mm)

Materials:

a) PDA media

b) Diesel fuel (1% and 10%)

c) Chloramphenicol (As a disinfection)

d) Distilled water

e) Acetone

Procedure:

Initially, the fungal strains were selected by assessing their growth potential in solid medium incorporated with diesel fuel. In order to determine fungal resistance, 1 cm^2 of 7-day old fungi colony on the agar palate was cut and were placed into the centre of 20 ml new fresh PDA plates with involvement of 1% and 10% (v/v) diesel (Akinnola, 2005; Shukor, 2013; Vanishree *et al*., 2014) dissolved in acetone (1:1 v/v). The PDA plates without diesel were preserved for negative control with each specified fungal strain (Lahkar & Deka, 2016). All palates were wrapped with laboratory parafilm and kept at room temperature for seven days. The radial growth assay by fungi colony on plates were verified and quantified by macroscopic feature during seven days inoculation using Vernier calliper measurement.

ii. Liquid Medium

Apparatus

a) Pipette (10 µl, 100 µl, 1 ml)

b) Pipette tips

c) Conical flasks (250 ml)

d) Compact cotton flask caps / plugs

e) Filter paper (42.5 mm, Whatman No.1)

f) Agitator (rotator)

g) Weighing balance

h) Vacuum pump (VCP8101) (500 mm Hg pressure)

i) Oven (UNB 400)

Materials:

a) Manufactured Potato Dextrose Broth (PDB): was (g/L): potato in fusion 4.0 (fusion from 200 g potatoes), D (+) glucose 20.0 g with pH: 5.1 ± 0.2 at 25 ºC, and was autoclaved at 121 ºC for 15 min.

b) Chloramphenicol (1000 µl/L) (As a disinfection)

c) Diesel fuel

d) Distilled water

Procedure:

Prior to biodegradation screening, all isolated strains which had performed effectively in solid medium with different petroleum hydrocarbons dosage were tested in PDB. Due to easy separation of filamentous structure of fungal, examining the capacity of preferred fungal strains to grow in liquid medium with presence of diesel was tested by quantitative filtration (Ferrari *et al.,* 2011; Kota *et al.,* 2014; Vanishree *et al.,* 2014). Therefore, fungal biomass was estimated as the constant dry weight of the mycelium. Twelve fungi isolates from different sources of soil were inoculated in conical flasks containing 50 ml PDB

incorporated with minimum 1% (v/v) and maximum 10% (v/v) (Akinnola, 2005; Shukor, 2013; Vanishree *et al.,* 2014) concentration of diesel fuel and 1000 µl/L chloramphenicol as disinfection. After autoclaving, two plugs of peripheral area of 7-day old pure fungal on PDA plates (1 cm^2 each) were added carefully. All inoculated flasks were kept at room temperature on 150 rpm constant shaking for 7-day biomass estimation of fungal strains (Godoy *et al.,* 2016; Yu *et al*., 2015). After growth period completion, fungal mycelia were vacuum-filtered through pre-weighted filter paper. All recovered fungal mycelia were dried at 75 ^{o}C oven temperature for 8 hours (Kota *et al*., 2014). They then were weighed again to obtain the dry biomass.

All experiments at this study were arranged by using 3×12 factorial (3 petroleum hydrocarbon dosage, i.e. Zero, one, ten, and 12 fungal strains). Two replicate inoculum flasks were preserved for each strain corresponding to two diesel concentration (1% and 10% (v/v)). The statistical data have been shown in all tables and bar charts. Flasks with devoid of diesel volume were employed as controls. At the end of this experiment, two assessments of fungal biomass Growth Inhibition (FGI) and Diesel Tolerance index (DTi) were conducted for selection of most tolerant fungal isolates. These assessments were calculated as the ratio of extended biomass of treated colony to that of untreated culture at PDB by using below equations (Akhtar *et al.*, 2013; Argumedo-Delira *et al.*, 2012).

$$\text{FGI}\ (\%) = \left(\frac{Wc - Wt}{W\,c}\right) \times 100 \quad \text{.....Equation 2}$$

$$\text{DTi} = \frac{B\,t}{B\,c} \quad \text{.....Equation 3}$$

Where FGI is fungal biomass growth inhibition, W_t is treated fungal biomass dry weight (mg), W_c is control biomass (without diesel dosage) dry weight (mg), and for third equation, B_t, B_c stand for dry biomass colony (mg) of treated with diesel and dry biomass

of control colony respectively. Fungal isolates with DTi value > 0.9, and FGI < 10% would be categorized as great tolerance to diesel fuel. Meanwhile, the DTi values < 0.9, and FGI > 10% can assorted as inhospitable agent.

Fungal growth assay data for dependent factor of biomass and substrate concentration as independent factor conducted at this study were subjected to One-Way ANOVA with single factor, following by F-test of two samples for variances and T-test of paired two samples for comparing the means. Differences with P-Value < 0.05 (P value = 0.01) or F > F crit (5.99 > 3.68) were provided sufficient evidence with 95% confidence level to consider statistically significant difference.

3.2 Testing of Diesel Biodegradation by Potential Isolates

The most tolerant fungal isolates to the diesel concentration were assessed through experiments including preliminary decolourization and GC/MS qualitative analysis to detect their ability of diesel removal as the target pollutant. The required criteria for the selection of fungal strains was they should not be classified as pathogens, and also they can re-produce in huge biomass in presence of diesel. Nevertheless, prior to biodegradation screening test, macroscopic and microscopic morphology of purred selected fungi colony were characterized for identification of ultimate strains.

3.2.1 Identification of Selected Fungal Strains

Apparatus

a) Olympus light macroscopy (S751)

b) Optica microscope binocular (B-150)

c) Glass microscope slides

d) Glass microscope cover slips

e) Needle

Materials:

a) Lacto phenol-picric acid

Procedure:

Identification of fungal strains were conducted by using association of colony macroscopic observations such as pigmentation and pattern, size and colour of colony growth, opacity, margin shape of colony, and colony surface texture; along with microscopic and morphological characterization (e.g. spores and hypha size and structure) based on the study conducted by Dhar *et al*. (2014). Microscopic identification was compiled by slide culture in lacto phenol picric acid wet mount compounded by Optica microscope in 10x, 40x and 100x oil lens as well as Optica Vision lite (1.2 Ink) software (Chaudhry *et al*., 2012; Gupta *et al*., 2012). Then fungal colony recognition to genera were conducted and compared according to established papers, web pages of fungal taxonomy key as well as pictorial atlas (Watanabe, 2010; Gupta *et al*., 2012).

3.2.2 Preliminary Diesel Biodegradation Test: Colorimetric Measurement

Apparatus

a) Erlenmeyer flasks (250 ml)

b) Agitator (rotatory)

c) Filter paper (42.5 mm, Whatman No.1)

d) Vacuum pump (VCP8101) (500 mm Hg pressure)

e) Pasteur pipettes (230 mm, 9")

f) Cuvette (one ml)

g) UV 2100 spectrophotometer

Materials:

a) Methylene blue ($C_{16}H_{18}C_1N_3S$) (redox indicator)

b) Tween 80 ($C_{64}H_{124}O_{26}$) (surfactant)

c) PDB

d) Chloramphenicol (1000 µl/L) (As a disinfection)

e) Diesel fuel (1% and 10% v/v)

f) Distilled water

Procedure:

Biodegradation capability of isolated fungi firstly was tested by employing the adjusted method base on the redox indicator according to the study by Al-Nasrawi (2012), Chaudhry *et al*. (2012), Ghosal *et al*. (2016), Menezes *et al*. (2017), and Koyani (2011). Two plugs of 7-day old fungal isolates (1 cm^2 each) were clipped from the edge of fungal culture on petri dishes and transferred into 50 ml sterilized PDB using 250 ml Erlenmeyer. According to study by Hadibarata and Tachibana (2009), medium with glucose carbon source has more affirmative effect on degradation rate of hydrocarbons compare to other sources such as sucrose. Additionally, 0.8 ml/L tween 80 (Behnood *et al*., 2014) and 2% v/v (0.05 ml/L) methylene blue with different diesel fuel concentration (1% and 10% v/v) were added to the liquid medium. All flasks were incubated at room temperature at 150 rpm rotatory agitator for one week (Yu *et al*., 2015). At first four days, flasks were sealed hermetically with laboratory Para film, after consuming all oxygen reservoir in flasks by microorganisms and resulting in produced gas (probably CO_2) at flasks shown on inflated Para films; plastic caps were riddled with holes finely to deserving O_2 for inoculum. During this setup, two flasks of controls were remained without inoculation at two different diesel concentration exposure. At zero, fourth and seventh interval days of inoculation, 2 ml of the inoculated medium were vacuum-filtered with paper filter, then cell-free aliquot were collected on two 1 ml cuvettes. Decrease in absorbance and colour changes from deep blue (initially) to colourless (finally) of inoculum with comparison of initial control flasks absorbance was captured on spectrophotometer at 600 nm

wavelength (Gargouri *et al*., 2015; Yu *et al*., 2015). Seedless broth medium including only nutrient medium was utilized for blank setup.

3.2.3 Confirmation of Diesel Biodegradation: by GC/MS

Apparatus

a) Erlenmeyer flasks (250 ml)

b) Round-bottom flask (RB flask)

c) test tube (15 ml)

d) Centrifuge (Z206A)

e) Separator glass funnel (250 ml)

f) Eppendorf centrifuge tubes (15 ml)

g) Glass wool

h) Glass funnel

i) Measuring cylinder

j) Pasteur pipettes (230 mm, 9")

k) Rotatory vacuum evaporator (N-1001SG-29) (rotavapor)

l) GC/MS vials (2 ml)

m) Gas Chromatography Mass Spectrometry (GC/MS) Agilent 7000 (triple quadrupole QQQ, 7890A)

Materials:

a) PDB

b) Distilled water

c) Diesel fuel (1% and 10% v/v)

d) η-hexane (C_6H_{14}) (solvent)

e) Anhydrous sodium sulphate (NA2SO4) (dryness reagent)

f) Chloramphenicol (1000 µl/L) (As a disinfection)

Procedure:

For fungal confirmation of actual diesel-utilizing fungal strains, all top-performed strains based on decolourization test were further examined for compound's characterization by GC/MS analysis. Fungal cultivating for GC/MS sample preparation was carried out similar to 3.1.3 section with different volume of liquid medium (20 ml) in 250 ml flasks (to reduce the usage of solvent). Flasks were supplemented with two different diesel concentration and were caped with compact cotton plugs in order to allow oxygen serving in for inoculum. Diesel fuel residual was extracted by using η-hexane for GC/MS analysis after preferred span of treatment (Yu *et al*., 2015).

i. Sample Extraction

Entire content of the Erlenmeyer flasks (inoculum) was poured into the 15 ml centrifuge test tube and after adding same volume of solvent (η-hexane) at 3000 rpm for 10 min was centrifuged. Cell-free supernatants (20 ml PDB+ 20 ml solvent) then was transferred to the 250 ml separator glass funnel. Organic phase of solvent was mixed by shaking vigorously for about 2 min with consequently ventilation to release gas. Followed by laying down the water phase for further 10 min and recovered into 250 ml culture flask; hydrophobic layer then was retrieve into clean flask. The aqueous was reprocessed twice with the extra 20 ml solvent (Farag & Soliman, 2011). Final 40 ml organic phase was filtered by passing through the glass wool and anhydrous sodium sulphate and was dried out with rotavapor coupled by 70 ^{o}C oil bath temperature. Later than total dryness, remaining diesel in the RB flask was quantified with η-hexane to reach enough volume of GC/MS vials. Latter, ending with about shaking for 1min, solvent was transferred into the vial for qualitative GC/MS analysis (Lahkar & Deka, 2016; Wu *et al*., 2010; Yu *et al*., 2015). Negative controls of the non-inoculated fungi medium were extracted using same extraction procedure.

ii. Instrument Condition

Gas Chromatography Mass Spectrometry analysis base on the method of Ameen *et al.* (2016) with minor modification was used. The GC was equipped by HP-5MS with a 30 mm - 0.25 mm ID (Internaldia) – 0.25 lm thickness (particle size) fused-silica capillary column. The GC/MS interface temperature was maintained at 280 ºC. Nitrogen as the collision gas and Helium was used as the carrier gas with flow rate of 1.0 ml/min. the oven temperature program was set as follow: 100 ºC hold for 1min, 15 ºC/min to 160 ºC at 5 ºC/min to 300 ºC hold for 7 min by 41 min each sample run time. The solvent delay time was set at 5 min and the GC injector was held isothermally at 280 ºC with maximum oven temperature at 325 ºC. As well as 1 µl of injection volume (Agilent Technology, 2014; Wu *et al*., 2010).

iii. GC/MS Analysis

GC/MS data analysis were conducted by qualitative analysis software incorporated by library version B.04.00/ built 4.0.225.0 software. Default area size detection was maintained at absolute area size ≥ 5000 counts and relative area size ≥ 1.000 of largest peak. Diesel biodegradation percentage (D %) then was obtained by the following formula:

$$D\ (\%) = \left(\frac{Ai - Af}{Ai}\right) \times 100 \quad \text{.....Equation 4}$$

Where A_i is the initial area size of compound peak and A_f is the final area volume of compound peak after fungal treatment (list of A_i and A_f for coresponding diesel concentration were given in the appendices A, B, and C).

3.3 Diesel Biodegradation in Higher Concentration of Biomass

Apparatus

a) Erlenmeyer flasks (250 ml)

b) Round-bottom flask (RB flask)

c) test tube (15 ml)

d) Centrifuge (Z206A)

e) Separator glass funnel (250 ml)

f) Eppendorf centrifuge tubes (15 ml)

g) Glass wool

h) Glass funnel

i) Measuring cylinder

j) Pasteur pipettes (230 mm, 9")

k) Rotatory vacuum evaporator (N-1001SG-29) (rotavapor)

l) GC/MS vials (2 ml)

m) Gas Chromatography Mass Spectrometry (GC/MS) Agilent 7000 (triple quadrupole QQQ, 7890A)

n) Bioreactor (1.5 L)

Materials:

a) Seven-day old fungi biomass

b) PDB

c) Diesel fuel (1% v/v concentration)

d) Chloramphenicol (1000 µl/L) (As a disinfection)

e) η-hexane (C_6H_{14}) (solvent)

f) Anhydrous sodium sulphate (NA2SO4) (dryness reagent)

g) Distilled water

Procedure:

A variety of studies have been supported the capability of petroleum hydrocarbon-utilizing indigenous fungi for degradation and elimination of the pollutants in the laboratory shake flask experiment size and condition recently (BK and SV, 2017; Husaini *et al.,* 2008; Mancera-Lopez *et al*., 2007; Mittal & Singh, 2009); but further performance confirmation in massive biomass is required, as microorganisms might not performing similarly as same as small-scale flask condition. Thus, the purpose of this study is to test the different isolate reactions in different concentration of biomass in fixed time. Therefore, further diesel biodegradation ability of prospected fungi in huge biomass were examined by using scaled-up condition in 1.5 L bioreactor applikon. The standard operation variable followed based on studies by (Godoy *et al*., 2016; Yu *et al*., 2015).This experiment were carried out at two stages as follow:

Stage I: Mass cultivation

Biomass cultivation of fungi isolates was carried out in 250 ml conical flasks containing 50 ml PDB supplemented with 1000 µl/L chloramphenicol. After autoclaving medium at 121 ºC for 15 min, two plugs of 7-old fungi culture which was performed well at flask shaking biodegradation experiment were added into the flasks. This experiment were conducted with ten flasks for each strain of isolate to get high concentration of biomass (roughly > 9 g/L). All flasks were kept at room temperature at 150 rpm constant agitating for one week.

Stage II: The Use of Bioreactor

In accordance with previous studies related to fungal life cycle for biodegradation efficiency, final 500 ml medium containing 7-day old inoculum in PDB was transferred into the 1.5 L glass stirred fermentation applikon. Liquid medium was adjusted into 1.5

ml and was then enriched with 1% (v/v) diesel fuel as the carbon source and was run for one week (Gargouri *et al*., 2015; Silva *et al*., 2015). In this experiment, fermenter batch was maintained at pH 7.0 (neutral condition) with stirring at 150 rpm and constant aeriation. According to Das and Chandran (2011) bioreactor temperature was set at 30 ºC for final reaching temperature of 28±1 ºC at surrounding the glass vessel. After 7 days, 20 ml of cell-free aliquot was picked from three depth of vessel to get stereotype representation of entire batch ambient. Same extraction procedure (referred to part 3.2.3) was applied for GC/MS sample preparation. Finally all samples were analysed by using GC/MS qualitative analysis. GC/MS data were analysed to get the pollution reduction percentage with similar formula of equation 4 which was employed in subsection 3.2.3.

CHAPTER 4: RESULTS

4.1 Primary Screening of Diesel Tolerant Fungi: Solid Media

Twelve strains were totally isolated from three different sources of the soil which are listed in the Table 4.1. Non-essential fungi which were not related to diesel degradation did not appeared during screening study using selective media. Table 4.1 also shows the physical morphology and origin of screened fungi (F-X – x denotes the serial number for the fungi isolate). Initial isolation of indigenous soil fungi have been carried out in PDA plates, in order to observe distinct morphological type of strains for characteristic identification. Besides, capability of fungi to tolerate diesel was observed based on the growth with presence of diesel substrate (different concentration). During the screening, all 12 strains of fungi have been observed grown on the selective media. This is in order to harness of the strain with growth capability in diesel exclusively on PDA plates.

Table 4.1: Macroscopic characteristic of 7-day old fungi isolates from PDA.

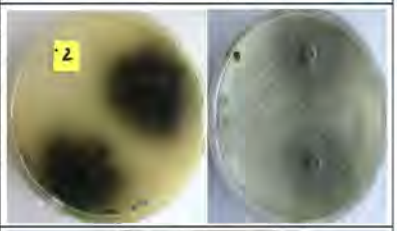
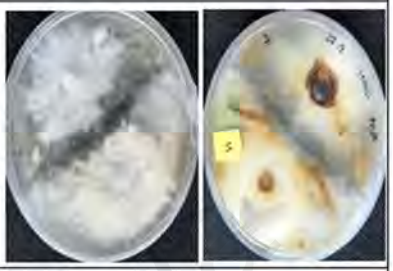
No.	Colony Code	Origin	Morphological Characteristics	
1	F-1	Run-off soil	Green at the centre with narrow white line at the edges/ moderate size/ irregular pattern and flat/ undulate and restricted margin/ wrinkled and dull surface also velvety with radius lines texture/ opaque opacity.	
2	F-2	petroleum contaminate area soil	Green at the centre and getting creamy white/ large size/ filamentous and flat form/ filamentous and fuzzy border/ velvety and dull surface/ translucent.	
3	F-3	petroleum contaminate area soil	Pinkish with radial of black moderate size/ irregular and radiate form/ flat in elevation / undulate and curled border/ watery and glistening texture and opaque.	

Table 4.1, continued.

No.	Colony Code	Origin	Morphological Characteristics	
4	F-4	Estimated natural soil	Creamy white from the top and tan at the canter on the bottom large in size/ Irregular pattern/ Crateriform in elevation/ Fuzzy and filamentous margin/ Cottony and fluffy surface and translucent.	
5	F-5	petroleum contaminate area soil	Buff/ Large in size/ Circular in border/ Raised elevation/ Spreading margin/ Velvety and Powdery texture/ Translucent in dimness.	
6	F-7	petroleum contaminate area soil	Dark green/ Punctiform(tiny) and Irregular / Flat in elevation/ Fuzzy border/ Velvety and Dull surface and Opaque.	
7	F-8 F-6	Estimated natural soil / petroleum contaminate area soil	Dark green with narrow white edge/ Small in size/ Circular, radiate and flat pattern/ Smooth an undulate margin/ Velvety, dull and opaque texture.	
8	F-9	Estimated natural soil	Dark green with white powder on top/Small size/Circular and convex pattern/ Smooth and entire border/Velvety, Dull and opaque texture.	
9	F-10	Run-off soil	Pale Olive green/ Moderate in size/ Irregular and flat form/ Undulate and restricted border/ Dull, velvety with radius lines and opaque.	

Table 4.1, continued.

NO.	Colony Code	Origin	Morphological Characteristics	
10	F-11	Run-off soil	Creamy white from the top and tan at the centre on the bottom/ Large in size/ Filamentous and Crateriform form/ Filamentous and fuzzy border/ Cottony, fluffy and translucent texture.	
11	F-12	Run-off soil	Creamy white from the top and tan at the centre on the bottom/ Large/ Filamentous and umbonate pattern/ Filamentous and fuzzy border/ Cottony, fluffy and Translucent texture.	
12	F-13	Run-off soil	Creamy white with black and white pigments/ Large size/ Irregular and Flat pattern/ Undulate and restricted border/ Dull, powdery and velvety surface/ Translucent in dimness.	

The fungi cultivated and cultured from three different origins were investigated to growth efficiency in solid media by growth diameter measurement in third, fifth and seventh day interval observation. Figure 4.1 shows sample of measurement for growth diameter in fungal colony. The diameter measurement was carried out to all twelve potential isolates, including control, 1% and 10% diesel (v/v). All of isolates were compared with the corresponding control without diesel substrate.

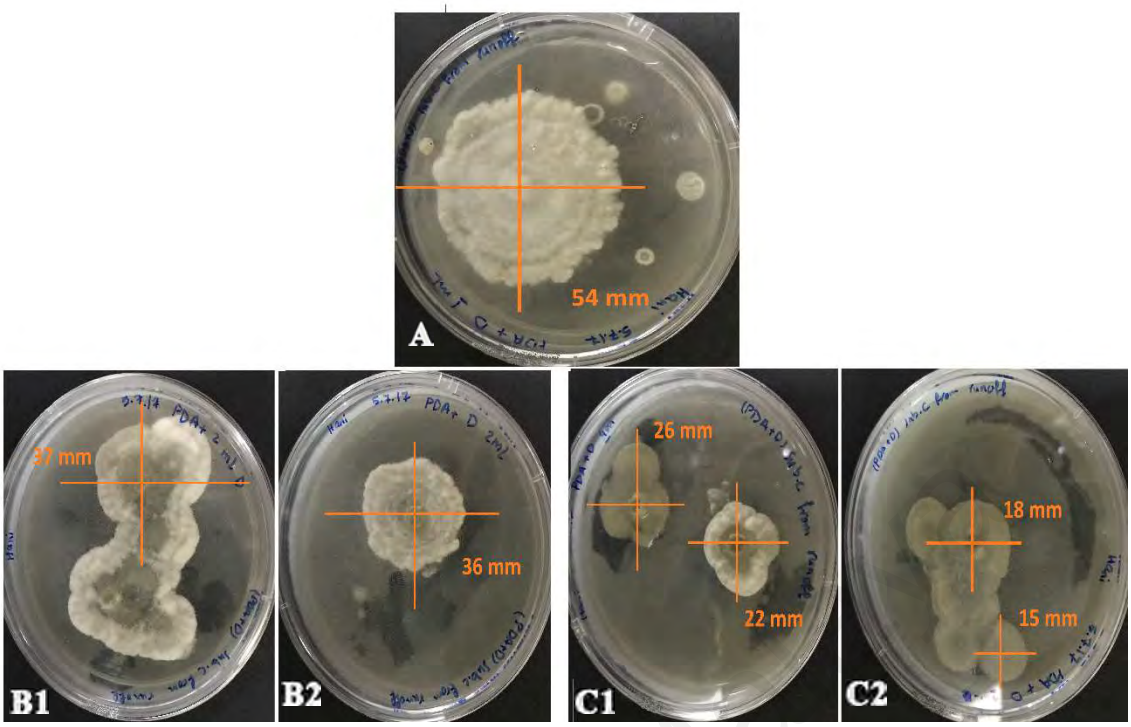


Figure 4.1: Samples of diameter measurement of growth assay for fungi culture colony after seven days.

*(A: Colony growth of control (fungi without diesel); B1 and B2: colony growth at 1% (v/v) diesel; C1 and C2: colony growth at 10% v/v diesel).

Figure 4.2 illustrates the comparatives diameter of all isolates at different concentration of diesel during seven days growth assay. All isolates except F-5 and F-12 have shown they are capable of growing at comparable rate for the assay of 1% diesel when compared to the control. Nevertheless, the growth were found to be much lower at 10% diesel assay. Moreover, the growth rate after third day of assay in 1% diesel for F-1 has exceeded the control plate without diesel. Generally, apart from F-12 strain, monitoring fungal colony growth of the most strains exposure to 1% diesel (v/v) at whole treatment period, did not represent the significant differences, but also fairly the same colony growth trend compared with their control plates.

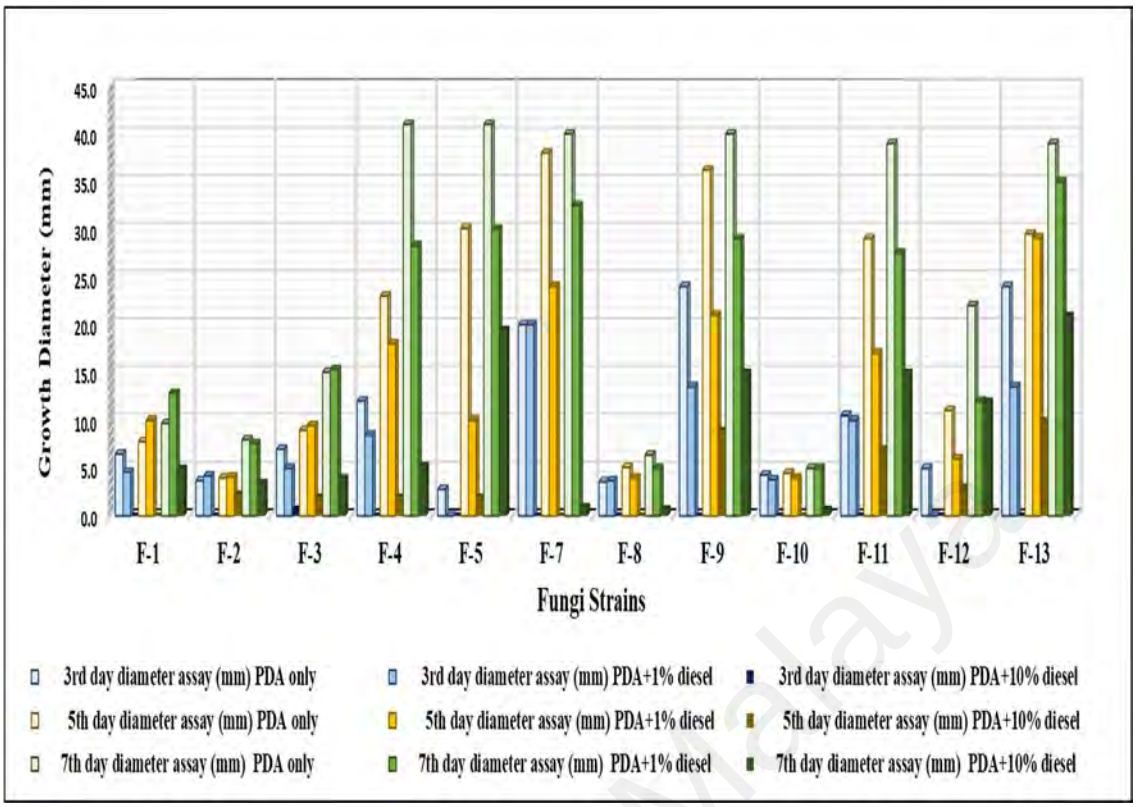


Figure 4.2: Comparison of fungal colony size and incubation period at different diesel concentration on PDA.

On the other hand, growth data of plates with 10% (v/v) diesel (v/v) showed no performances before day three. Although diesel did not notably inhibit the fungal growth after seven days; high concentration of diesel might have the detrimental effects on the filamentous growth of all 12 fungal isolates. Some effects on the mycelium overflow, radial pattern, and sporulation sparsity of fungal species were observed which could be the element of growth inhibition and/ or attacking the fungal mycelia around the diesel oil (halo formation) which was dispersed on agar plates.

Induction of growth rate was obtained by increasing the treatment period for all three deserved diesel quantities (zero, one and ten percent) (Argumedo *et al*., 2012), which was remarkable in some cases with coverage of all agar surface at seventh day, without and with 1% diesel substrate. The similarity of growth size of most isolates was observed at the end of treatment period.

4.2 Secondary Screening of Diesel Tolerant Fungi: Liquid Media

All twelve colonies of fungal isolates were cultured in PDB flask shaking, for the investigation of isolates fungi growth in liquid media. Figure 4.3 shows the fungal biomass growth of strains in two different diesel concentrations in selected broth and control for seven days. Adaptation of fungal isolates to this study at diesel toxicity is referred to their growth rate, which was measured by its biomass concentration within specific time (Kota *et al*., 2014). Almost all isolates in 1% diesel show similar growth rates, when compared to control. While, for 10% diesel the growth rate were found to be lower. Nevertheless, at both concentration, the growth of fungi still took place. The more increases in treatment period of time leads to the more fungal biomass concentration (data not provided).

It has been detected that out of twelve, five species of fungi, i.e. F-1, F-3, F-7, F-8 and F-13 have shown the great accumulation of biomass especially with higher diesel fuel concentration compared to their corresponding controls without diesel. These results are also confirmed from the DTi and FGI (%) analysis in Table 4.2 at two different substrate concentrations. The values from FGI (%) < 10% and DTi > 0.9, demonstrate the tolerance and growth of all twelve isolates at one percent diesel fuel. Meanwhile, respecting to the FGI (%) and DTi rates at 10% diesel oil, only five out of twelve isolates, i.e. F-1, F-3, F-7, F-8 and F-13 have met the cut of point of FGI (%) < 10% and DTi > 0.9. Moreover, it was discovered that with the majority of fungi species the volume of biomass at flasks with one percentage of diesel as well as the strains namely, F-1, F-8, and F-13, the growth rate of fungal with 10% (v/v) of substrate took over in comparison with the control flasks of zero diesel fuel concentration. Besides, only at these remarked three strains, FGI percentage of (-) 70.77, (-) 1.78 and (-) 1.83 showed the negative values respectively; and for DT index of 1.71, 1.02 and 1.02 obtained the bigger than one (> 1) respectively. This indicates that these strains be able to utilize diesel as carbon nutrient and resist high

concentration of diesel fuel more than the rest. Therefore, the FGI (%) and DTi analysis can use for screening most resistant fungal isolates at high diesel oil contamination.

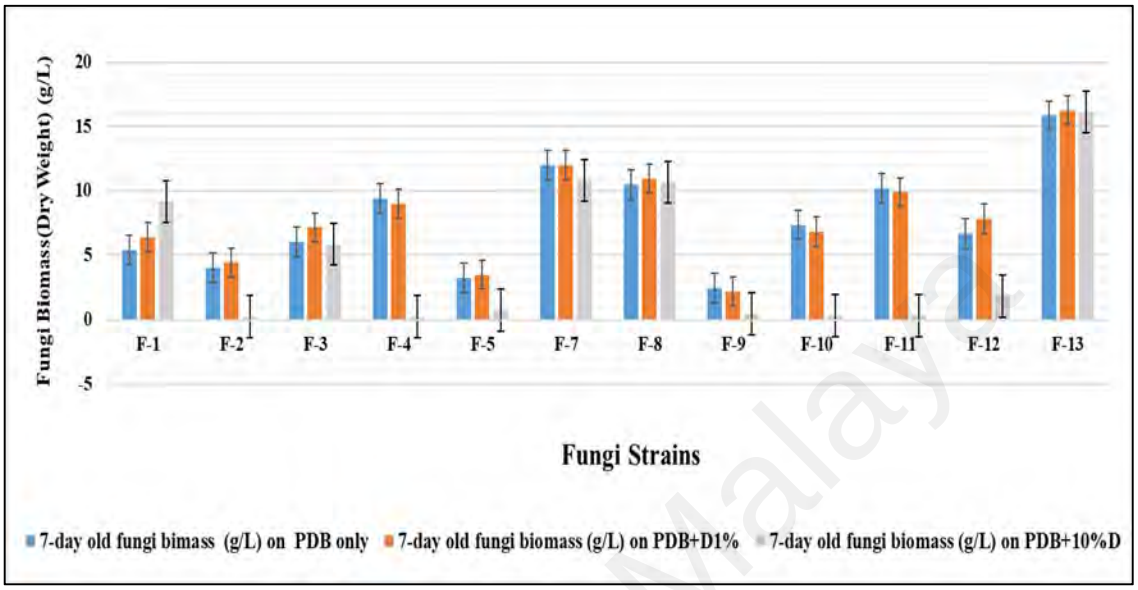


Figure 4.3: Dried biomass of fungal colony in different diesel concentration in PDB. *("D" Represent the diesel substrate)

Table 4.2: List of isolates for FGI and DTi at 1% and 10% (v/v) diesel substrate.

Fungi isolate	FGI (%)		DTi	
	1% (v/v) diesel	10% (v/v) diesel	1% (v/v) diesel	10% (v/v) diesel
F-1	(-) 19.05	(-) 70.77	1.19	1.71
F-2	(-) 10.56	95.02	1.11	0.05
F-3	(-) 18.41	3.48	1.18	0.97
F-4	4.40	97.69	0.96	0.02
F-5	(-) 6.87	77.48	1.07	0.23
F-7	(-) 0.15	9.62	1.00	0.90
F-8	(-) 4.54	(-) 1.78	1.05	1.02
F-9	10.15	81.20	0.90	0.19
F-10	7.28	95.72	0.93	0.04
F-11	3.35	96.88	0.97	0.03
F-12	(-) 17.54	72.79	1.18	0.27
F-13	(-) 2.72	(-) 1.83	1.03	1.02

*(Negative values indicate the lack of inhibitory)

*( ) Represents the most tolerant strains at high diesel doses.

F-13 gained the maximum growth of 16.12 g/L followed by F-7 and F-8 of 10.82 and 10.63 g/L respectively in 10% diesel exposure; whereas, minimum biomass growth were conducted with F-2 and F-4 of 0.2 g/L. All five top- performed species (i.e. F-1, F-3, F-7, F-8, F-13) from the secondary screening in PDB then retested again on same shake flask condition, for confirmation of selected fungal tolerance with different concentration of diesel in liquid media. The results from this experiment also support the finding from previous one. Figure 4.4 divulges the gravimetric test outcomes of all preferred fungi culture in different diesel oil dosage. Repeating experiment revealed that F-3 has the least growth rate contrasting to the rest four culture colony with very colloidal filamentous structure at liquid media. In addition to what has already been remarked at previous experiment results, growth rate at all five preferred strains arises in present of one percent of diesel concentration in contrasting to the control flasks with zero dosage of diesel. Moreover, Table 4.3 of FGI (%) and DTi values assessment for each acquired discerned isolate at two various diesel quantities indicate the similar outcomes as well, which shows the lack of growth inhibitory of low and high diesel concentration with selected strains.

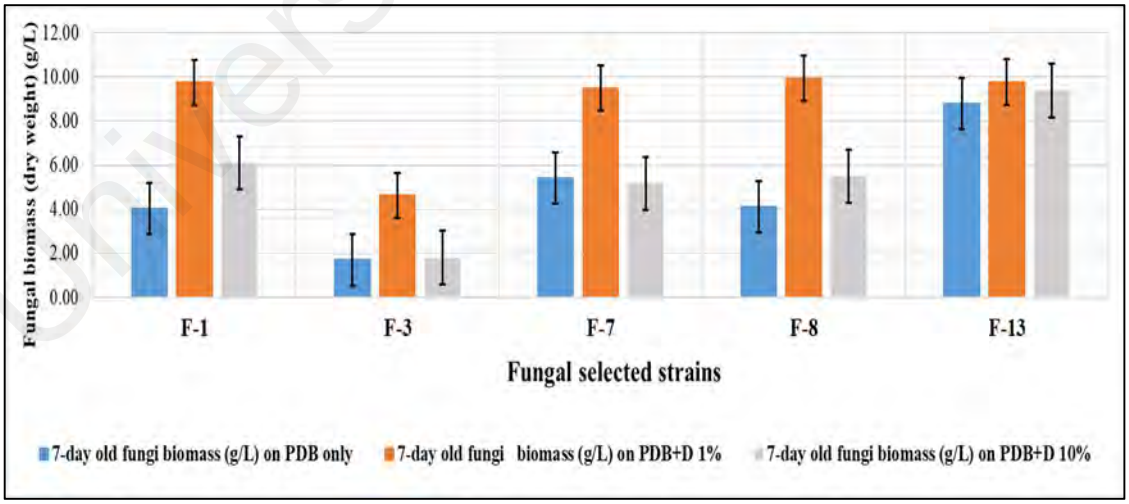


Figure 4.4: Comparison dried biomass of selected isolates in different concentration of diesel exposure.

*("D" Represents the diesel oil)

Table 4.3: Tabulation of FGI and DTi at 1% and 10% (v/v) diesel substrate for selected fungal isolates in PDB.

Selected Fungi isolate	FGI (%)		DTi	
	1% (v/v) diesel	10% (v/v) diesel	1% (v/v) diesel	10% (v/v) diesel
F-1	(-) 142.14	(-) 51.44	2.42	1.51
F-3	(-) 172.88	(-) 6.97	2.73	1.07
F-7	(-) 75.01	4.79	1.75	0.95
F-8	(-) 141.00	(-) 33.06	2.41	1.33
F-13	(-) 11.03	(-) 6.66	1.11	1.07

*(Negative values indicate the lack of inhibitory)

4.3 Identification of Selected Isolates

Five isolates, based on their good affinity to grow in presence of diesel in the screening test, were selected for identification up to species level. The identification of strains were critically carried out through microscopic and macroscopic observation with standard key of soil and seed fungi. Figure 4.5 exhibits the macroscopic and microscopic characteristics of preferred fungus strains. Due to undefined feature of mycelia and hypha structures of some isolates, the isolates were stained differently at 40X and 100X of magnification. The comparison of five isolates with the standard key revealed that, all genus belonged to *Ascomycota* phylum. The isolates of five, closely related strains as *Penicillium* sp.A*, Aureobasidium* sp., *Penicillium* sp.B*, Penicillium* sp.C, and *Aspergillus* sp. (Figure 4.5 and Table 4.4).

Table 4.4: List of diesel-tolerant fungi with their source of isolation.

Fungi Isolate Strains	Cultural Colony Codes	Origins
Penicillium sp.A	F-1	Run-off soil
Aureobasidium sp.	F-3	petroleum contaminated area soil
Penicillium sp.B	F-7	petroleum contaminated area soil
Penicillium sp.C	F-8	Estimated natural soil / petroleum contaminated area soil
Aspergillus sp.	F-13	Run-off soil

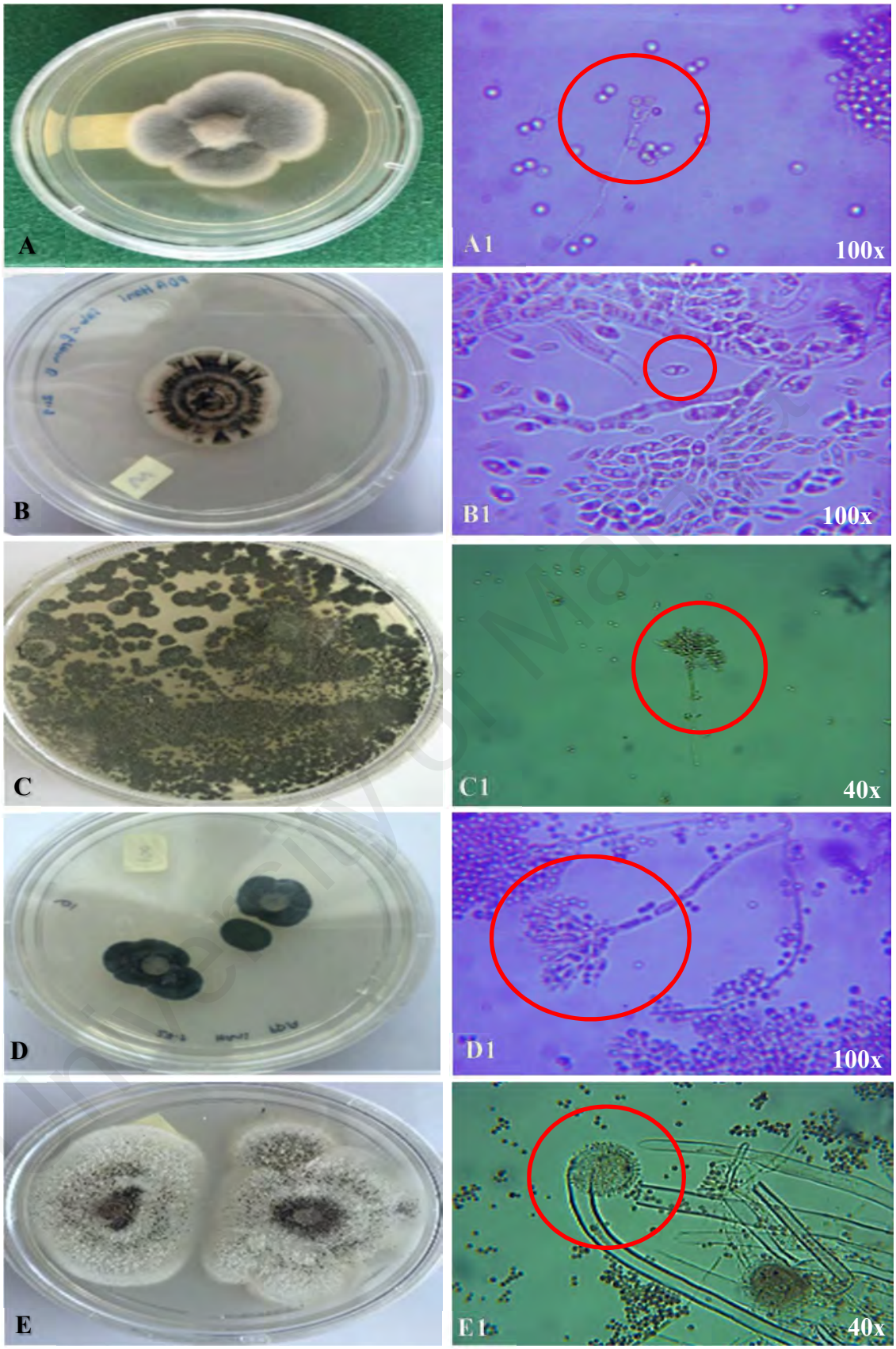


Figure 4.5: Photograph of macro and micro of preferred isolates. A, A1: *Penicillium* sp.A (F-1); B, B1: *Aureobasidium* sp. (F-3); C, C1: *Penicillium* sp.B (F-7); D, D1: *Penicillium* sp.C (F-8); E, E1: *Aspergillus* sp. (F-13).

*(Red circles represent the microscopic specification of mycelia with each strain.)

4.4 Biodegradation Study Using Liquid Media

Confirmation of diesel biodegradation efficiency of selected fungi strains based on the screening in liquid media was firstly conducted using redox indicator for colour transformation from deep blue to colourless. Later the biodegradation of diesel was analysed using qualitative gas chromatography mass spectrometry analysis.

4.4.1 Preliminary Test of Diesel Biodegradation: Colorimetric Measurement

During preliminary test of indigenous selected fungal for biodegradation ability, all *Ascomycota* species were evaluated for their culture media colour changes. This work was based on the colour changes which could be observed in some flasks. Whereas, this rate of transforming in some flasks occurred to lesser extent. Control flasks were referred to liquid medium without fungi inoculation. Figure 4.6 indicates the light absorbance mean values for five selected fungal strains with different diesel oil dosage at initial, forth, and seventh day of treatment. No measurement was taken before day four, because there was no color changes. From the Figure 4.6 it is discovered that the wavelength mean value of all examined fungal strains showed the reduction, which is signifying in exposure of high diesel concentration. The least absorbance of 0.265 nm observed of medium treated by F-13 with presence of 10% (v/v) contamination, which indicates highest diesel biodegradation ability. While, high value of 0.886 nm wavelength observed at medium inoculated with F-7 by 1% diesel (v/v) indicates lowest ability of diesel degradation.

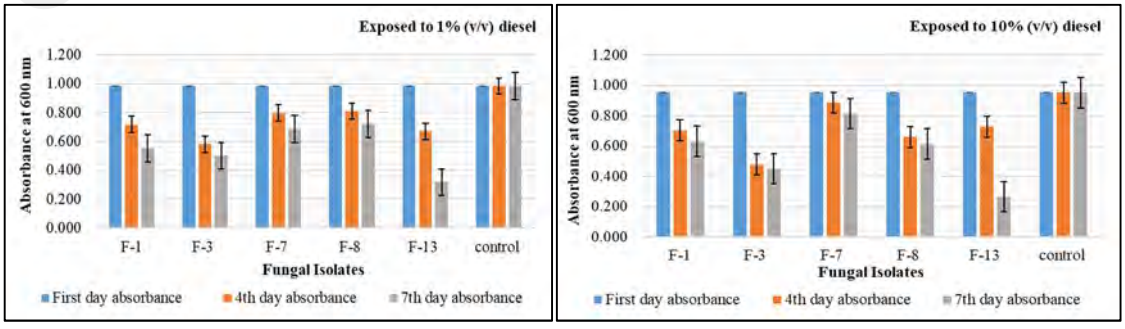


Figure 4.6: Comparison of absorbance for selected fungal in different diesel concentration.

Results of optical density measurement are shown in Figure 4.7 for *Penicillium* sp.A (F-1), *Aureobasidium* sp. (F-3), and *Aspergillus* sp. (F-13). They represented fastest and highest inception of decolourization (reduction in absorbance value) of 43%, 49%, and 68% with 1% diesel (v/v) respectively. Similar trend of decolourization at 34, 53, and 72% were shown by 10 (%) diesel (v/v). the least inception of decolourization of less than 30 % was observed in medium cultivated by *Penicillium* sp.B. (F-7) with both concentration of diesel. Therefore, conducting of highest decolourization inception have revealed the capability of hydrocarbon degradation in selective isolates.

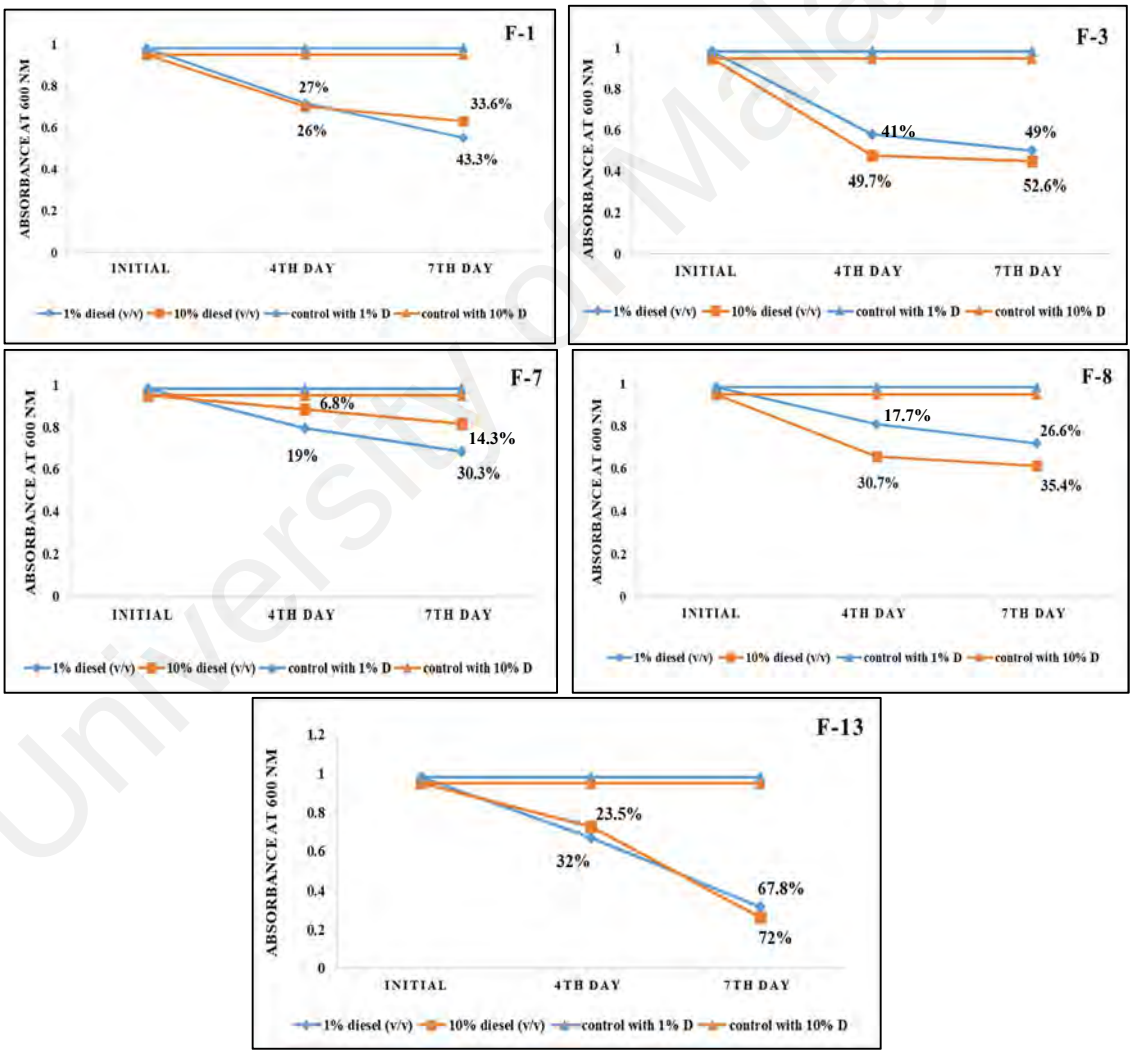


Figure 4.7: Optical density of PD broth media treated by five selected fungi isolates.

(Indicates for different diesel concentration at initial, 4th, and 7th interval day of observation.("F-X": colony code; "D": diesel))

4.4.2 Confirmation of Diesel Biodegradation: by GC/MS

Biodegradation capacity for all five selected fungi strains were confirmed using gas chromatography coupled mass spectrometry. Diesel degradation was only focused in this study and final diesel concentration was not measured. Reduction of diesel concentration was measured based on the detected peak at time zero for the control media; and compared to the peak at day seven for the media inoculated with five prospect fungi. Figures 4.8 - 4.9 show the chromatograms of GC/MS for both 1% and 10% (v/v) diesel concentration undergoing biodegradation study using all five potential isolates.

The establishment of peaks' sequence were recorded with the similar functional trend at all experimental tests with only alteration on compounds and apparition in new different peaks. Diesel fuel chromatography columns revealed that about 70% of diesel mass is aliphatic fractions, where only 20% is consist of aromatics and the rest is consist of sulphur, nitrogen, oxygen, and additives. This is in agreement with what Mukherji *et al.* (2004) found in his study for biodegradation of diesel by microbial colony from deep-sea sediments. Generally, the majority of high peaks were detected by NIST library program belong to saturated aliphatic hydrocarbons such as n- alkanes (e.g. nonane, decane, and eicosane); as well as Iso- alkanes up to C_{28} (i.e. non- aromatic rings such as cyclooctyle and cyclohexanone), and in lesser extent aromatic compounds for instance naphthalene (i.e. two benzene rings). Fragmentor voltages for these composites were eluted from range of 5 to 31 min acquisition time with varying from C_{11} to C_{28} which represented the number of carbon atoms in compound composition of diesel.

The diesel substrate profiling chromatographs at 1% (v/v) concentration for five selected isolates at zero and 7 days are shown in Figure 4.8 (diesel profiling of substances respecting to area size in detected peaks and retention times are given in Tables A.1 in Appendix A; Tables B.1-5 in Appendix B). A quick scan on the spectrum for all samples

when compared to control, had manifested some degree of diesel reduction. Meanwhile, we found the moderate reduction on peaks related to n-alkanes compounds such as eicosane (to be appeared at the end of acquisition time sequence) and hexadecane (paraffin) (detected at low retention time). Aromatic hydrocarbons such as mostly naphthalene and its related compound classes were recorded on the beginning of sequences' detecting peaks which seem to scale down in abundances intensively; but not so many existence of devaluation of compositions. For instance, the naphthalene to be detected in the peaks of 5.2 and 6.4 min acquisition time at control, were remained the same compound in the treated diesel profile with only reduction on the concentration.

Based on the qualitative analysis for diesel residual on medium treated by F-1 and control, for extreme degree, hydrocarbons profiling demonstrates the breaking down of complex branched carbon compounds, which belong to n-alkanes at standard sample into the simple linear chained carbon structures. For instance, substances such as dimethyl cyclohexanon and ethyl octahydropyrano [3, 2-b] pyridin were discerned at control compound list did not characterize in loading compound profile for diesel degradation ability test (for both two different concentration) of *Penicillium* sp.A (F-1).

Hydrocarbon compound analysis of diesel constituent inoculated with all *Penicillium* species (i.e. F-1, F-7 and F-8) revealed the almost same reduction rate and trend in area magnitude mean values. However, diesel degradation ability of F-1 and F-8 in presence of low contamination charges were documented in greater rate of 70 %; whereas, this amount with F-7 showed the lower rate of 60.7% respectively (The variation of degradation and reduction percentage for two different diesel substitutes respecting to time of detection for each isolate was given in Table D.1 in Appendix D).

GC/MS chromatogram analysis for diesel profiling treated with *Aureobasidium* sp. (F-3) depicts the great reduction of more than 85% in compound peak volumes as comparing

to control. Maximum drop in peak highs and area size for pollution treatment with F-3 strain were monitored for middle of retention time with parallel to other diesel profiling chromatograms of fungal strains performance.

However, inoculated one (%) diesel oil volume with *Aspergillus* sp. (F-13) compare to no inoculated flask control showed the most outstanding decrease of up to 89.2 % in the area size of remarked peaks among the other experiments. This is indicating collapse and/or lessen of fundamental compounds. Identified peaks belonging to mostly Iso-paraffin and naphthene groups were reduced up to great extent with low pollution load of diesel by *Aspergillus* sp. (F-13). Not only component loading of diesel treated with this strain (F-13) showed the greatest reduction in the area abundance corresponding to n-alkane groups such as octene and nonane and Iso-alkanes such as dodecycloxy and cyclohexanone; but also, performed the satisfactory drops in the two or three ringed aromatic hydrocarbons concentration which is mostly being naphthalene.

To summarize, although the decreases in the compound peak area did not follow the same trend at inoculated medium by all five selected isolates; entire range of n-alkanes on the diesel hydrocarbons profiling, from nonane (C_{11}) to hexacosane (C_{28}), were degraded in general.

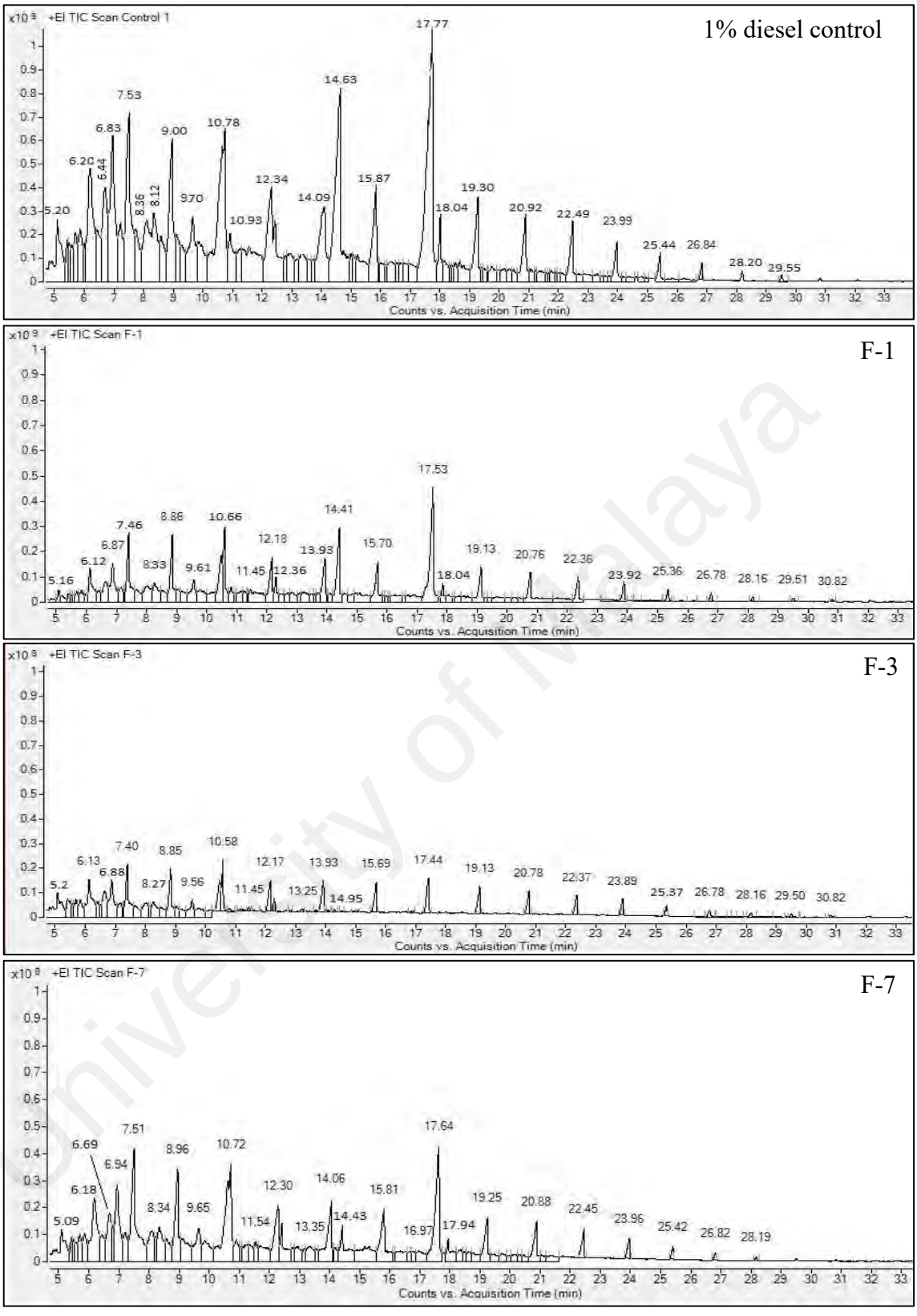


Figure 4.8: Chromatograms of diesel profiling by GC/MS analysis for control with 1% diesel volume, *Penicillium* sp.A (F-1), for *Aureobasidium* sp. (F-3), *Penicillium* sp.B (F-7), *Penicillium* sp.C (F-8), and *Aspergillus* sp. (F-13).

*(Top numbers of peaks represent the retention time value (min) for spotted compounds)

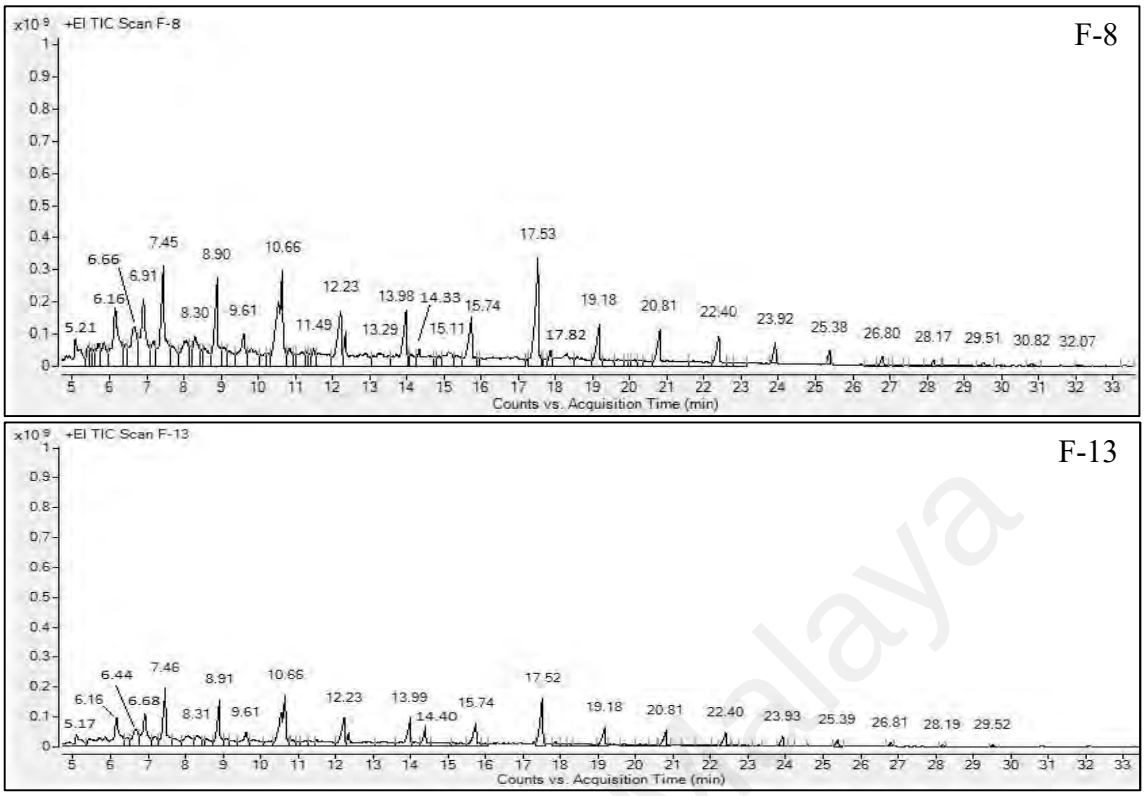


Figure 4.8, continued.

All shake flask experiments with five selected isolates were also tested for 10% diesel concentration. The diesel residuals were then decomposed into component elements by GC/MS analysis in order to obtain the degradation rate. Chromatograms analysis of degradation experiment at 10% diesel concentration due to overlapping the peaks and high concentration perceived by GC/MS was not discernible; thus, all samples were diluted in 10^3 X factor. The latter values were multiplied in dilution factorial. Though, there was a elevating in area size of identified peaks with extension of diesel oil concentration, yet the significant disintegration has been seen on priority constituents of diesel. According to the chromatograms at higher concentration of diesel, Compounds elution were occurred between 5 to 32 min of retention time.

Data analysis of initial and final volume peaks of diesel residuals for bio-degradation efficiency of all five selected isolates showed the reduction. Area size of peaks for diesel residual treated by F-1 revealed the highest removal percentage of up to 70.6% of diesel.

However, this mean value did not make more changes in the inoculated flasks with rest of the isolates (diesel profiling of substances respecting to area size in detected peaks and retention times are given in Table A.2 in Appendix A; Tables B.6-10 in Appendix B).

The mean values of 68% reduction with 10% of substrate was conducted from abundance of peaks analysis treated by F-3, F-7 strains; which represents the similar breaking down of diesel compounds. Nevertheless, hydrocarbon compound analysis of 10% diesel chromatogram treated with F-8 revealed the reduction rate in area magnitude of 66.3%. Meanwhile, degradation competence of *Aspergillus* sp. (F-13) at high concentration of diesel pollution did not represent the similar removal rate as compare to low dosage; which was up to 63.9 % after one week incubation period. It showed the least level of degradation with high diesel concentration (The variation of degradation and reduction percentage for two different diesel substitutes respecting to time of detection for each isolate was given in Table D.2 in Appendix D).

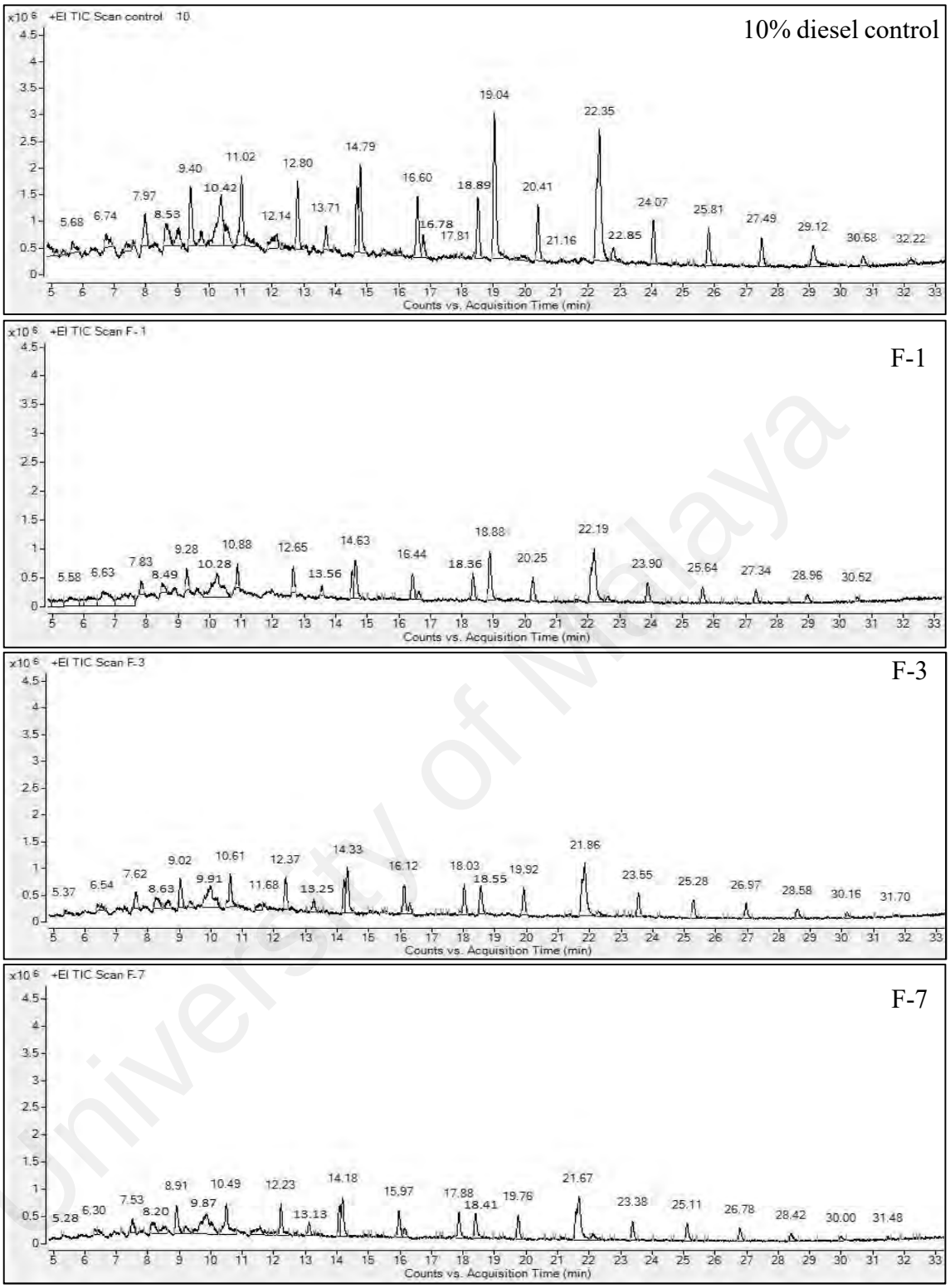


Figure 4.9: Chromatograms of diesel profiling by GC/MS analysis for control with 10% (v/v) diesel volume, *Penicillium* sp.A (F-1), for *Aureobasidium* sp. (F-3), *Penicillium* sp.B (F-7), *Penicillium* sp.C (F-8), and *Aspergillus* sp. (F-13).

*(Top numbers of peaks represent the retention time value (min) for spotted compounds by GC detector. Data values for 10% diesel concentration must be multiplied at 10^{3}).

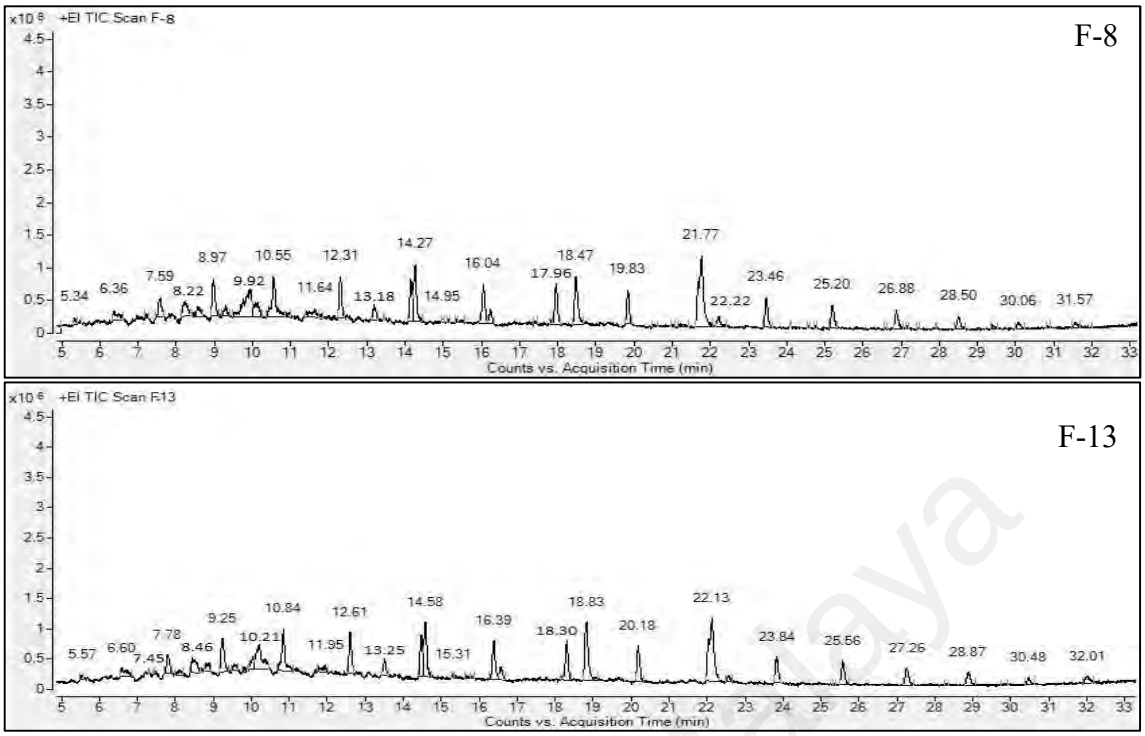


Figure 4.9, continued.

To deduct, almost similar removal area percentage of 70% with slight differences of peaks selection were reported for both concentrations of substrate treated by *Penicillium* sp.A (F-1). However, it was discovered that this degradation range with other two strains of *Penicillium* sp.B (F-7) and *Penicillium* sp.C (F-8) were not in the same trend, where with increasing in substrate concentration F-7 acted oppositely, and showed more reduction on area size of detection peaks. Meanwhile, based on the chromatogram area size, the mean value of diesel reduction at 10% volume degraded by F-3 represented a slight falling down on degradation rate compared to low concentration. This comparison for the flasks seeded by F-13 at both diesel concentration remained the same trend of dropping in the degradation rate by increasing the toxicity level.

The large portion of aromatic compounds (substantially naphthalene class) seemed to diminish with all selected isolates at minimum and maximum diesel fuel oil volumes, but not break down and altering the parent aromatic carbons to simple fusions.

4.5 Diesel Biodegradation Assay in Higher Concentration of Biomass

Out of five, two top-performed fungal isolates of *Penicillium* sp.A and *Aspergillus* sp. from two different genera were selected for further diesel biodegradation study in high concentration of biomass using bioreactor. The selection of isolates was carried out based on the highest diesel reduction capability at different concentration of substrate. Based on the seven-day bioreactor experiment with high volume of biomass, both two prospect isolate's induction in filamentous of fungi cultures were observed with increasing of treatment period. Figure 4.10 shows the fungi biomass accumulation on bioreactor applikon at the end of the treatment.

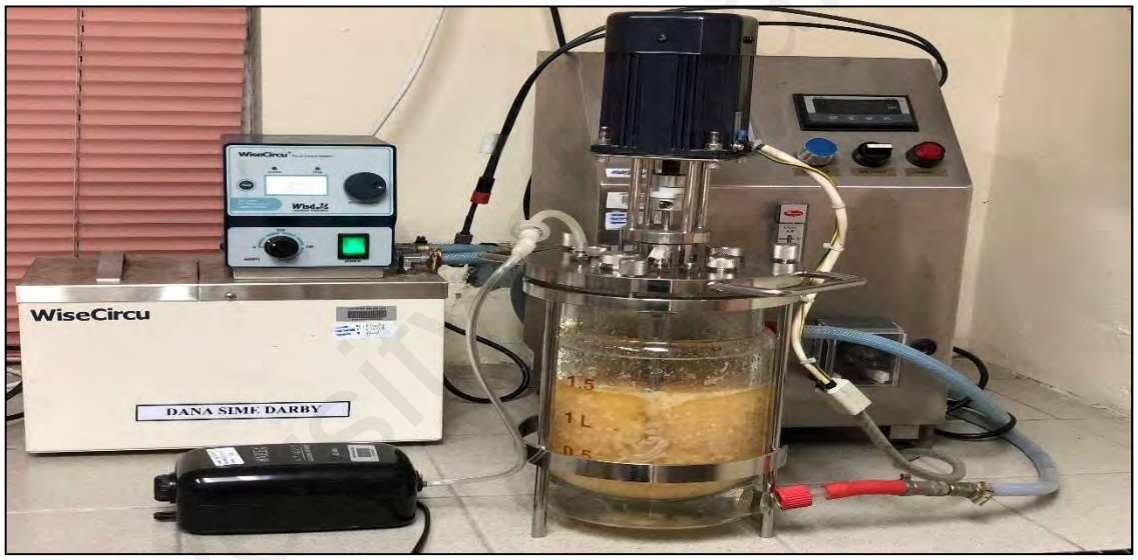


Figure 4.10: Experimentation set up for up-scaling the bioremediation of diesel.

*(Figure presents the bioreactor vessel and control panels for aeration, agitation and temperature adjustment)

4.5.1 GC/MS Analysis

Gas chromatography method with mass spectrometry detection of diesel profiling chromatograms treated with two prospected strains of *Penicillium* sp.A, and *Aspergillus* sp. and corresponding control at initial stage of low dosage treatment are shown in Figure 4.12. Broader range of hydrocarbon compounds elution of the acquisition time of 3 to 35 min were recorded by NIST library program.

In the beginning phase of chromatograms (Figure 4.12), disappearance of some peaks were completely conducted through qualitative evaluation of the compound area size of detected peaks. For instance, the peaks with retention time of 5.06, 5.55, 5.82, and 6.6 min which are standing for n-alkanes and aromatic (with one carbon ring, i.e., benzene) compounds were vanished after one week of biodegradation by *Penicillium* sp.A strain. Meanwhile, this complete degradation of compounds by *Aspergillus* sp. were documented at 5.06, 5.55, and 6.6 min of retention time with seven days bioreactor treatment (diesel profiling of substances respecting to area size in detected peaks and retention times are given in Tables C.1-3 in Appendix C).

Despite the fact, this changes to total removal condition were not found in the shake flask experiments, but the total degradation rate comparatively remains nearly the same with slight increase, up to 77.3 % for *Penicillium* sp.A strain (The variation of degradation and reduction percentage for two different diesel substitutes respecting to time of detection for each isolate was given in Tables D.1 and D.2 in Appendix D). Figure 4.11 presents the comparison of total diesel component degradation percentage with two selective fungi strains of *Penicillium* sp.A and *Aspergillus* sp., through shake flask and bioreactor trials. Differentiation in the volume of the discerned peaks at higher concentration against the control samples and shake flask experiment represented the considerable fall-offs of up to greater than 41% in the removal ratio of diesel fractions treated by *Aspergillus* sp.(F-13) after seven days. Therefore, in higher concentration of biomass, the degradation of diesel components was not as good as in shake flask. This indicated the life span of the subjected enzyme were shorten compared to other species (Al-Hawash, *et al*., 2018).

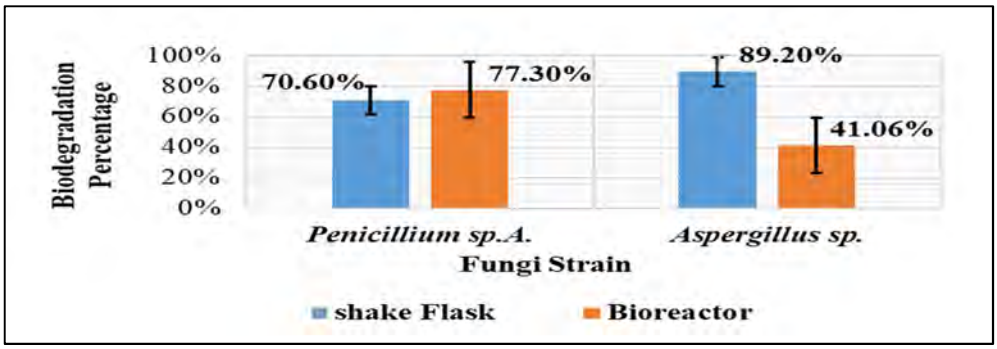


Figure 4.11: Comparison of biodegradation rate in two test condition with prospect fungal isolates.

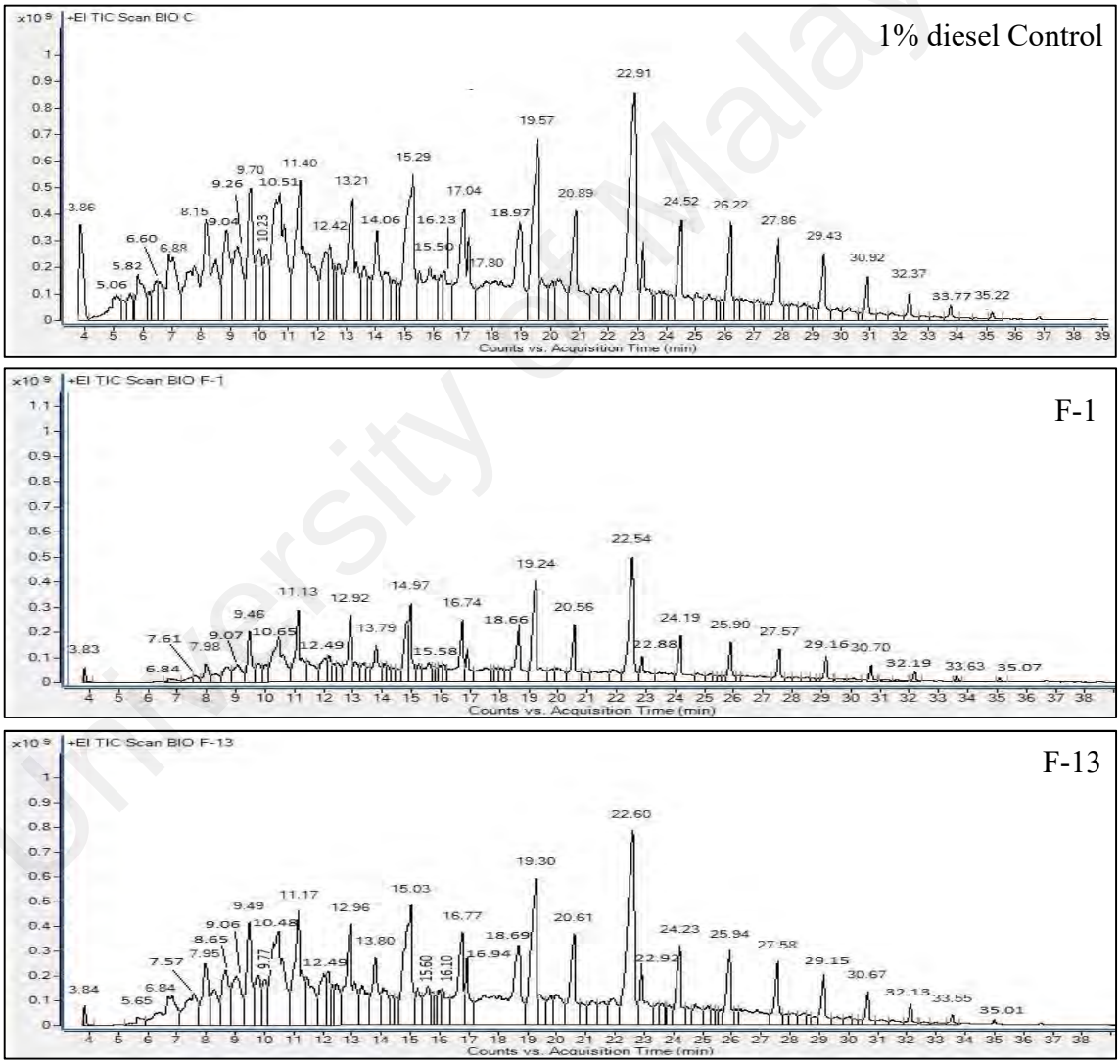


Figure 4.12: Chromatograms of diesel profiling by GC/MS analysis for *Penicillium* sp.A (F-1), *Aspergillus* sp. (F-13), and control with 1% (v/v) diesel volume in bioreactor applikon.

*(Top numbers of peaks represent the retention time value (min) for spotted compounds by GC detector).

CHAPTER 5: DISCUSSION

This part of study elaborates the fungal isolates identification and their general performances. Diesel tolerance and degradation efficiency of selected isolates at this study have discussed according to different concentration of substrate, incubation time and experimental conditions.

5.1 General Discussion about Diesel Tolerance of Fungi

From recent studies, it is intensively reported variety of indigenous microorganisms such as fungi in previously exposure to hydrocarbon contaminations of water and soil are capable to tolerate and survive in extreme environmental condition. Native fungi could degrade broad range of organic pollutants (Kues, 2015; Maddela *et al*., 2015). Putting into other words, contamination can alter favorably the characteristic and biodiversity of natural microorganisms which can tolerate polluted condition (Mohsenzadeh *et al*., 2009; Zafra *et al.*, 2015). Therefore, indigenous fungal community will be the best choice of degradation agent selection (Atagana *et al.*, 2006). Thus, prior to satisfactory degradation, efficiency evaluation and tolerant assessment of naturally-occurred fungi would be the promising method for cultural preference.

According to Grosberg *et al.* (2012) terrestrial fungi comprise the substantial ecological group of fungi. Comprehensive studies on petroleum hydrocarbons tolerance using fungi isolated from different structural soil, water and sediment samples of contaminated and undisturbed sources have been documented. Nevertheless, hardly any studies have been conducted on diesel tolerance and degradation by fungi isolated from the soil to apply in liquid media (Table 2.4).

5.1.1 Overall Performance of Identified Fungi

In this study, during co-cultivation fungi with one percent diesel fuel proved that mostly all isolated species showed the acceptable growth which can be effect of optimum main nutrition sources to grow; on the other hand, with increasing the concentration of diesel in flasks, the filamentous growth rate in some extent was significantly decreased with these colonies (Figure 4.3). This is in agreement with Vanishree *et al.* (2014) which reported decreases in the biomass production of *Penicillium* sp., i.e. isolated from petrol and automobile workshop, towards increases in crude oil concentration. This biomass reduction could be the result of hydrocarbons pollution and toxicity expansion. Furthermore, these results are in similarity of Hadibarata *et al.* (2007)'s study that reported growth halting in *Trichoderma* sp. S019 from forest sources with higher concentration of substrate in liquid medium supplemented with phenanthrene. However, some isolates in Figure 4.3 has been shown the more tolerance to high volume of diesel oil comparing to low volume of substrate and even without contamination (F-1, F-8 and F-13). This is in accordance with the study by Mohsenzadeh *et al*. (2009), which proposed the fungi especially *Fusarium* sp. with biodegradation potential in highly crude oil polluted soil even greater than that in lower volume at semi-arid arias.

Therefore, we can state that different diesel sensitivity values could be obtained with each distinct fungal isolates. This differences might be the results of various adjustment actions taken by each strain of isolates in relation to pollution toxicity. This opinion strongly supports the above description.

5.1.2 Identification of Indigenous Fungi

According to previous research, fungi tolerance and growth rate evaluation suggested as the best tool to select the proper strain to be employed as biodegradation agent (Dos Santos *et al*., 2008; Ulfig *et al.*, 2006). Moreover, by reason of co-culturing all twelve

isolates at this study in PDA incorporated with diesel oil, they are partially tolerant to diesel substrate. Despite the fact that solid agar medium is more suitable for fungi growth and sensitivity observation which reported by Zapotoczny *et al.* (2007); regarding to diesel stationary condition and solubility affection in solid agar environment, final strain selection carried out in liquid medium monitoring right after growth assay assessment in solid medium additionally. This is because various cultural and growth responses to different dosage of contamination in selected liquid medium compared to growth tendency in solid media have found at present study; which is in similarity to the report by Taboski *et al.* (2005). Therefore, for ultimate selection of highly-tolerant strain the estimation of highest (> 0.9) DTi and lowest (< 10%) FGI were employed at high diesel concentration in liquid media after testing all targeted strains in PDA.

Not all twelve isolates belonging to same genera have the similar diesel tolerance capability. Three strains from genus *Penicillium* sp. (F-1, F-7 and F-8) isolated at this study was discovered, tolerated to diesel fuel in different trend. For instance F-1 responded positively to ten percent diesel in contrast to presence of one and zero volume of diesel, while the two rest acted in opposite way. Dos Santos *et al*. (2008) stated the diverse colony growth rate (ranging from 2.1 to 0.8 mm/day) of four strains classified in *Aspergillus* sp. at 0.25 and 0.5 % (w/v) diesel concentration. This can be explain as a fact that each fungal strain have particular enzymatic activity and inhibitory adaptation mechanism (Argumedo-Delira *et al*., 2012).

Several researches considered on bioremediation rate and hydrocarbon-utilizing of these fungi isolates ability as well. For example, the selection of isolates of *Penicillium* sp. and *Aspergillus* sp. that exhibited the high capability to acquire petroleum hydrocarbons in the form of diesel fuel characterized equally in the previous study by Ferrari *et al*. (2011). Moreover, Dighton and White (2017) have been revealed the

degradation function of complex hydrocarbons especially in hypersaline condition by all three species, i.e., *Aureobasidium* sp., *Penicillium* sp. and *Aspergillus* sp. isolated at this study. However, irrespective to high diesel assimilation ability, we disregarded the *Aureobasidium* sp. due to the extreme colloidal sporulation in liquid media, as well as, the report of high pathogenicity of this isolate for human and plants reported by Bakermans (2015). Therefore, we examined the other two strains of *Penicillium* sp.A and *Aspergillus* sp. for further biodegradation study under bioreactor condition.

To summarize, according to dos Santos *et al*. (2008) *Aspergillus* sp. can purified from contaminated soil and is the one of the several fungi isolates could tolerate the broad range of contamination. Besides, the fact that *Penicillium* sp. at this study, also can bear the contamination source condition which was confirmed in several previous studies such as Maddela *et al.* (2015) and Vanishree *et al.* (2014).

5.1.3 Measured Variables during Biodegradation

Factor of fungal strain with specific enzyme production and metabolism is one of the most major elements which can influence the rate of pollution tolerance ability by fungi rather than its origin of isolation. Base on the results in present study, *Penicillium* sp. C (F-6/ F-8), i.e. isolated from petroleum workshop and undisturbed soil did not show the outstanding distinction, and in actuality remained the same outcomes. Same outcomes were conducted from prior research by Atagana *et al*. (2006) which revealed that there was no significant differences between *Pleurotus* sp. performances isolated from contaminated source and that from un-contaminated soil. Furthermore, in report of Rodríguez-Córdova *et al.* (2016), three species of *Penicillium* i.e., isolated from different sources in peatlands and Magellan soils contaminated with petroleum products could tolerate high concentration of hydrocarbons in similar trend.

Though during the diesel treatment period, significant difference, obtained from ANOVA one way and T-test at P-value of 0.01, in dry biomass of fungal isolates has been observed at various volume of substrates. There was a direct correlation between growth rate of filamentous fungi and treatment period. Based on the baseline data in Figure 4.3 which has been represented the increase of fungal biomass. The fungal biomass induction could be the result of bioavailability of carbon nutrients in forms of organic pollutant to survive and continue cell production; as well as, to promote biodegradation process in fungi culture (Iheanacho *et al.*, 2014). This statement has been reviewed in study by Sebiomo *et al.* (2011) which reported the expansion in dry weight values by time span with the number of fungi isolates from mechanic soil samples during utilization of crude oil and gasoline.

5.2 Degradation of Diesel by Indigenous Fungi

This section discus about potential of indigenous soil fungi from different soil sources to degrade diesel in liquid medium. Terrestrial native fungi cultivated in huge biomass in limited time of laboratory condition. They would have filamentous structure for trapping the contamination, as well as owing unspecific extracellular enzymes, that are advantageous for the biodegradation process. Fungi are known to remediate hydrocarbon pollutants serve as carbon source by their enzymatic metabolisms, more effective than other microorganisms such as bacteria and algae. Several studies have been conducted to investigate the best biodegradation representative to mitigate organic pollutants such as petroleum hydrocarbons (Table 2.4). Regardless of genera type of fungi, many other elements can influence the biodegradation capacity of targeted fungi, factors such as environmental conditions, i.e., temperature, pH, and pollutant composition, bioavailability and concentration of pollution and many more. Fungal biodiversity and their correlation respect to other microorganisms also can alter the process of biodegradation (Varjani & Upasani, 2017). This study was only investigated the

prospected indigenous fungi isolates ability to biodegrade diesel fuel oil in two different concentrations.

In our study, out of twelve, five prospected fungal isolates based on their feasible biomass germination and relatively lesser inhibition growth rate approaches at high diesel volume, examined for their substrate removal capability by redox indicator decolourization and GC/MS qualification. They are belonging to *Ascomycota* division from *Penicillium*, *Aspergillus* and *Aureobasidium* species. In this experiment, biodegradation capacity of fungal isolates calculated in terms of total reduction on detected compound peaks in diesel chromatogram profile of GC/MS and loss of total diesel contamination did not quantify. For GC/MS, there was no specific component determined, because the study is a qualitative study to search for indigenous fungi ability to degrade diesel.

5.2.1 Evaluation of Diesel Biodegradation

This part of discussion is based on the absorbance value conducted through redox methylene blue technique (initial diesel biodegradation process). All prospect fungi isolates in this study had able to utilize diesel as a carbon source relatively. Total color changes from deep blue to colorless at the end of fungi inoculation time suggest that these isolates produce oxidative enzymes, possibly the hydrocarbon oxidizer (Iheanacho *et al*., 2014). Strain *Aspergillus* sp. (F-13) showed the fastest and highest color reduction in both 1% and 10% (v/v) diesel concentration. These findings are also in agreement with the report of Chaudhry *et al*. (2012) within variation the same values for *Aspergillus* spp. and *Penicillium* spp.. Allude to the data plot, in Figure 4.6, reduced absorbance values indicate higher degradation rates due to either the percentage of oxidizing hydrocarbons, or/and fungal biomass growth which use oxygen for enzymes metabolism (Gupta *et al.*, 2012), thus all five fungal colony could have partially this property.

Results of our GC profiling expressed that as initial concentration continue to increase from zero (%) to 10 % (v/v), the biodegradation capacity of isolates decreased corresponding to different species. However, the two exceptions obtained for strains of *Penicillium* sp.B and *Penicillium* sp.A in which area size reduction percentage were increased (up to 68%) and remained same (comparable of 70%) respectively. *Penicillium* sp.B (F-7) showed 7 % more reduction in the value of the peaks' area volume by higher diesel quantity; meanwhile, *Aspergillus* sp. (F-13) reacted of a contrary kind to pollution expansion of 25% decreases in degradation rate. In similar studies by Chehregani Rad *et al.* (2014); Mohsenzadeh *et al.* (2012) reported the degradation rate of hydrocarbon by *Penicillium* sp. and *Aspergillus* sp. decrease from 55% to 20% as the concentration of petroleum hydrocarbon in forms of diesel and crude oil was increase from 2% (v/v) to 10% (v/v). However, the reduction in degradation rate at high volume of diesel were also noticeable; which indicated the fact that reported earlier by Chaudhry *et al.* (2012) of diesel-biodegrader fungi adaptation to high load of hydrocarbon which is differ in degree and quality with each strain of fungi.

5.2.2 Fractionized Diesel during Biodegradation

Definite identification of the degraded and used compounds is not feasible, due to the complexity of diesel (Marchand *et al*., 2017). Though, aromatic fractions particularly naphthalene (consist of 2 benzene rings) and substances with more than 2- benzene rings proved to be resistance, as they re-appeared in seeded microbial flasks residuals in lesser volume. Therefore, complete exploitation of high weight hydrocarbon fractions in diesel by fungi for metabolic process seem not to be accomplished. Moreover, according to Winquist *et al.* (2014) aromatic compounds formed with < 4-ring degraded slightly in laboratory flasks treated by fungi, but hydrocarbons with > 5- benzene rings of those remain near to the same amount. Similar result was reported by Atagana *et al*. (2006) of partially utilization of naphthalene by all tested fungal strains. Nevertheless, Mittal and

Singh (2009) in their study on crude oil biodegradation by *Aspergillus niger* revealed that fungi are efficient but slow in aromatic fractions (more than 60 days).

Overall observation for both Figures 4.8 and 4.9 shows that during the seven days of treatment by selected potent fungal, the major portion of substance's removal is in n-alkanes fractions (short linear aliphatics), and also the complex components, i.e., aliphatics and aromatics in the lesser extent. Thus, based on the peak's area, biodegradation of hydrocarbon by all selected fungi are in a sequence, started with, n-alkane followed by branched alkane with more carbon chain, low molecular weight of aromatics and cycloalkanes which were the least vulnerable to strike. This arrangement is also reported by Vieira *et al.* (2007) on the study of diesel and gasoline; except that they are used bacteria, not fungi.

Based on diesel composition profile, fatty acids (such as tridecanoic acid and octadecanoic acid) occurrence in seeded microbial culture are the end-products of degradation procedure of hydrocarbons (Table B.1-10 in appendix B). This is in agreement with the studies by Silva *et al.* (2015) and Sebiomo *et al.* (2011) which reported the slight acidity condition in the treated flasks by fungi comparing to initial time of treatment. They revealed that filamentous fungi through aerobic degradation process can alter n-alkane to fatty acids. This fact also exhibited at study by Hadibarata and Tachibana (2009) on n-alkane degradation using *Trichoderma* sp. S019 into fatty acid (nonadecanoic acid) which considered through analytical derivatization process. Saratale *et al.* (2007) also reported that the presence of two fatty acids (i.e. aldehyde and carboxylic acids), as the dead-end products of kerosene (hydrocarbon) biodegradation by *Aspergillus* sp.

New compound peaks' occurrence (e.g. Heptadecane, Azulene, Docosane, and Tetracosane) in residual of treatment flasks depicted the degrading and breaking down of some corresponding parent compounds. However, the toxicity of diesel oil was reduced

after one week at all five treated flask shaking experiment in some extent. Base on area size of detected peaks of substance, the highest reduction mean values of 70.6%, 85.6%, and 89.2% were found with strains of *Penicillium* sp.A (F-1), *Aureobasidium* sp. (F-3), and *Aspergillus* sp. (F-13) respectively with one (%) diesel inoculation flasks treatment. These values were greater compared to *Penicillium* sp.B (F-7) and *Penicillium* sp.C (F-8) which were able to degrade 60.7% and 70% of diesel respectively.

5.3 Diesel Biodegradation Using High Concentration of Biomass

Biodegradation of diesel using high concentration biomass of fungal isolates was conducted to monitor and evaluate the degradation process in 1.5 L bioreactor. High biomass concentration of two strains of *Penicillium* sp.A and *Aspergillus* sp. which showed higher degradation ability of diesel, were selected for this experiment. The highest degradation rate of diesel oil was obtained in the bioreactor batch treated by *Penicillium* sp.A (F-1) with 77.3% peaks removal of major diesel substrates; while *Aspergillus* sp. showed the 41% reduction in peaks area, respectively at 7^{th} day of incubation.

In this study, the biomass profile of the isolates was visually observed to constant increases with prolong of experiment time (data not provided). This could be the indication of promoting degradation progresses were carried out by inoculum and obtainable nutrients in the medium (Iheanacho *et al.,* 2014). Actually this similar observation has been reported earlier for biomass induction of consortium with presence of 10% diesel fuel during seven days bioreactor trial (Silva *et al.,* 2015).

In present study, diesel removal percentage were estimated by GC/MS qualitative analysis. Based on the diesel chromatogram load, both fungal isolates (mentioned earlier i.e. F-1 and F-13) are owner of the biodegradation potential in the higher concentration of biomass using bioreactor. Fungi strains tested in bioreactor showed reduction in most

constituents' peaks of diesel at the beginning phase of chromatogram; these peaks mainly detected as saturated n-alkanes and single-ring aromatics. Therefore, total quantity of microorganisms will contributes to bio-removal promotion of initial substrate. Our findings from bioreactor test are an agreement with previous studies of fungal ability to degrade alkane fractions (Vanishree *et al*., 2014). However, findings at this study are the prospect of indigenous isolates being tested in higher concentration of biomass to degrade diesel. While, other studies used consortium for the diesel biodegradation study. In addition, others used other substrates such as crude oil instead of diesel.

5.3.1 Comparison to Shake Flask Incubation

Comparing to the findings from this experiment with those on shake flasks at similar volume of diesel, *Penicillium* sp.A (F-1) represented the nearly same reduction value of more than 77%. Generally, according to recent study by BK and SV (2017) during bioreactor trial, the increase in inoculum of isolate will lead to increase in biodegradation rate which is clearly supports the result of diesel degradation by F-1. However, there has been 48% drops on the reduction rate by *Aspergillus* sp. under bioreactor condition. This could be due to the variation in fungal proliferation and diversity of lifecycles through different classes of fungi as an entomopathogen which leads to kinetic enzyme activity and organic matter degradation in different time laps with each kind of strain (Dighton & White, 2017; Wu *et al.,* 2010). Thus with this case, biodegradation probably took place at first phase of fungal life cycle which is before seven days (not because of the experimental errors due to duplicate performing). This is in agreement with the result from study by Saratale *et al.* (2007) on the 10% (v/v) kerosene detoxification ability of *Aspergillus ochraceus* in PDB*,* the constant increases has been observed for the growth rate of dried biomass weight after six days incubation; meanwhile, the oxidase enzyme productivity and kerosene biodegradation activities have been decreased during this time. Furthermore, maximum enzyme production, and as a consequence, maximal

contamination oxidation rate have been reported at the day fourth with this species. Therefore, the optimum productivity of *Aspergillus* sp. takes place at different specific time phase comparing to *Penicillium* sp..

Although the factors on pH and mixing speed were not tested at this study; still it was observed that constant agitation factor can multiply connection between semi-liquid mixture phase and gas phase composed of oxygen and volatilized substrate in the flask shaking. As a consequence, the more loss of substrate occur by volatilization process the more increases would be in the removal rate. Whereas, this situation will not happen in the hermetically-closed bioreactor applikon (Hadibarata *et al*., 2007; Marchand *et al*., 2017). Therefore, the difference in biodegradation percentage values in shake flask with that on bioreactor applikon is the result of volatilization process of the lower molecular weight components in the diesel fuel.

Last but not least, employment of huge fungal biomass through the bioreactor process could be favorable for higher bio-removal achievement with some selected fungal isolates. However, using higher biomass of fungi for biodegradation process has shown that did not exhibit the consistent reduction of diesel. Different fungi isolates react differently.

CHAPTER 6: CONCLUSION

This study is important, because it will provide eco-friendly way of removing PH contamination in the hydrosphere. Other than bacteria, fungi is also one of the agent, which could be used to remove the contaminant. Contamination of water by PH is equally as threatening as others. It may cause spread and sediment in the water. Although a lot of literatures focusing on the removing of PH by the spillage, this study attempt to be used for industrial waste water treatment prior of discharged into the environment (ex-situ treatment). The acquired data in this study is sufficient for the detailed discussion, tough not enough for in-depth.

Our study managed to isolate and screen 12 strains that are able to degrade diesel in liquid media, within the rage of concentration of 1% to 10% (v/v). Use of the diesel is to represent the PH. These 12 isolates were obtained from three type of soils, namely, approximately near natural soil, runoff soil, and petroleum contaminated area soil. It was found that, there is no direct relationship between groups of fungi with the type of soil. The diversity of fungi isolates seem not dependent to the condition of the sampling sites, i.e., isolates F-6 and F-8 (*Penicillium* sp. C) can be found in two different sites (estimated natural soil and petroleum contaminated area soil). However, discussion for the soil fungi is not the primary subject, because the study intent to obtain potential isolates from any possible sources, not particularly from soil.

All isolates were screened through selective media of 1% diesel (v/v) substrate. The use of solid media with targeted pollutant would assist to narrow down the number of isolates, eliminating the least diesel tolerant isolates. Screening with solid media provide better understanding for the distinction of isolates based on its morphology within same genus. Notwithstanding the fact that, the cultivation of isolates in solid agar is the

imperative technique for fungal growth and sensitivity screening; it is also overcoming the difficulties of stationary condition and solubility of diesel in solid phase.

Twelve selected isolates were further tested by growing them in selective liquid media. Similar trend of growth in solid agar were observed in latter media. The results from fungal inhibitory growth rate in solid and liquid media revealed that one (%) v/v diesel contamination is the low toxicity level for the selected strains. Moreover, it is likely to stimulate the fungal mycelia growth after seven-day incubation time. Meanwhile, high loading of diesel had indicated the inhibitory and detrimental effects on fungal growth. However, in general, growth of 12 isolates either in solid or liquid media increased with prolonged inoculation time for all deserved diesel concentration. This indicates that fungal mycelia and its enzyme metabolisms were going through the adaptation to the stressor (diesel). The following experimentation for FGI and DTi tests, examined in duplication, have shortened down to five isolates that are able to withstand high concentration of diesel (10 % v/v). They are F-1, F-3, F-7, F-8 and F-13.

In the state of advanced equipment absence (such as PCR method of DNA sequences), the morphological characteristic of isolates in solid agar can be also used successfully for fungal colony's identification up to the genus level, which is implemented in previous studies as well. Generally, out of twelve, five effective indigenous fungi isolates as a diesel hydrocarbons degradation agent have been observed belonging to *Ascomycota* division, i.e., *Aspergillus* sp., *Penicillium* sp. A, *Penicillium* sp.B, *Penicillium* sp. C, and *Aureobasidium* sp. isolated from different soil sources.

Earlier studies have addressed that the biodegradation of diesel by fungi are limited by their inhibitory growth rate during the process. Meanwhile, this study observed that there is a possibility that some isolates may behave differently. For example, the studied strain *Aspergillus* sp. (F-13) could not thrive well up to low concentration of substrate, while

the biodegradation ability of this isolate was the highest rate of 89% among the other isolates with the same volume of pollution. On the other hand, *Penicillium* sp.B (F-7) which is the least tolerant isolate is found able to degrade 68 % of diesel concentration which is considered a fair degrader.

Expected results of actual fungal biodegradation potential can be quickly conducted through preliminary qualitative decolourization test. However, the results of redox indicator test (decolourization) are not as accurate as GC/MS analysis. Although, GC/MS is the comprehensive way of analysis to yield the expected precise results for each component of substrate; the use of standards for the recognition of each component in a complex substrate such as diesel has known to be the limitation for the GC/MS analysis. In this study, it was found that dilution the sample is a good way to minimize noises from chromatogram profiling for 10 % diesel. It was done on the treated samples.

Results of GC chromatograms in this study did not demonstrate a consistency behavior in both concentrations of substrate. Degradation of long-chained hydrocarbons can be expected to occur over a longer period of incubation time by all selected five isolates at different rate. Other than shake flask experiment, this study also used higher concentration of biomass to biodegrade the diesel. We have found that a) elevation of biodegrading rate and b) decreasing the degradation rate of diesel by both selected strains of *Penicillium* sp.A and *Aspergillus* sp. respectively. With both findings, it can be postulated that different strains will give different reactions at various condition. Therefore, more study need to be done to identify the optimum condition and also the life span. The break-down of hydrocarbon by higher concentration of biomass still is the similar to low concentration, which is not consistent.

To conclude this study, it can be inferred that usual method of isolation and screening are capable of obtaining to highly potential isolates to degrade PH. Moreover, both

Aspergillus sp. and *Penicillium* sp. strains can be employed for PH degradation in higher concentration of biomass.

6.1 Future Recommendation

Respecting to what we have found and conclude, the number of limitations should be consider for future experimental work:

- Present study assembled for exploring the diesel biodegradation ability of some preferred fungal isolates in primary stage, which we employed the commercial diesel composition. However, for fulfilment of biodegradation approaches which is an application implemented for mixed contamination removal in the environment; hence, the efficiency of diesel-utilizing fungal isolates should be further practiced and considered in massive biomass under natural field condition. Due to the fact of differences between laboratory and ex-situ circumstances may not convey the true applicability of experimental findings in-vitro.
- Although the numbers of research have reported on n-alkanes and aromatic hydrocarbon compounds-utilizing fungi from different sources in diesel composition. However, due to complexity of diesel, better understanding of which compounds or fractions to be used by fungal enzymatic metabolisms and its degradation pathway are required. Further study to investigate these capability on tropical indigenous fungi should be also carried out.
- Misidentification or incomplete coding (PCR identification method) of prospected microbial strain which has been hardly apply in environmental researches is also one of the major drawbacks of in most petroleum biodegradation studies. This has led to uncertainty and inefficient implementation strategies and evaluation in-situ bio-decontamination.

- Meanwhile, concerning about sustainability and eco-friendly possessions of native fungi strain, the huge biomass of appropriate use of indigenous fungal culture should be contemplate for other probable impact to ecosystem. For instance, Mishra and Nautiyal (2009) reported that augmentation of *Trichoderma ressei* to diesel contaminated soil has the antagonistic effects on other microbial residences' population.
- Several studies have been done on investigation of pure microbial culture ability either for fungal community or bacterial colony for organic pollutant removal from soil and liquid medium. None of those was found capable of total degrading all constituents in the complex mixture of petroleum fuel by itself. It is suggested that using consortiums including fungi and bacteria will lead to more efficiency in hydrocarbon biotransformation and removal. Therefore, utilizing both indigenous cultural colony in the form of bacteria and fungi isolated from tropical ecosystem should be explored in order to answer enquiries about synergistic effects of microorganisms and greater metabolic interaction approaches for pollutant removal.

In overall, according to broad range of fungi mechanisms and activities in the natural ecosystem with complex mixture of contamination, more researches regarding to investigate and find out the degradation potential of proper native fungal species which can completely eliminate the organic pollutants in ex-situ, in-situ or pilot study yet needed.

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