FACULTY OF INDUSTRIAL SCIENCES & TECHNOLOGY (FIST)
UNIVERSITI MALAYSIA PAHANG

FINAL REPORT FOR GRANT NO.:

RDU160333

TITLE:

BIOETHANOL PRODUCTION FROM DISPOSED ENGINE OIL MANAGEMENT

START DATE:	25/05/2016
END DATE:	24/11/2018

Prepared by:

PROJECT LEADER:

ASSOC. PROF. DR. ESSAM A. MAKKY

Click here to enter text.

ABSTRACT

Disposed engine oil (DEO) is a recalcitrant carbon source for microorganisms as it is mainly composed of long chain saturated hydrocarbon. Hydrocarbon-degrading microorganisms from oil contaminated soil sample were successfully used for bioremediation of DEO. This research aims to study and bio-convert DEO and DEO contaminated soil to lipase enzyme and ethanol, which is common industrial waste that contain heavy metals which are harmful to environment and human health. Also to utilize and isolate microorganisms that is capable to degrade DEO and find the optimum parameters for the best lipase and ethanol production. Lipase production GS-3 bacterial isolate was successfully isolated from oil contaminated soil area. GC-MS analysis revealed that this isolate was able to produce organic acid, methyl-3, 4, 5-trimethoxy-2, 6-dinitrobenzoate from disposed engine oil. Besides, among the five solvents screened to extract the hydrocarbon present in the oil contaminated soil sample, toluene has the highest efficiency up to almost 0.041(µl/ml) of hydrocarbon which represent 93.3% of hydrocarbon from the sample. Therefore GS-3 isolate produced highest lipase activity, achieving 0.097 ± 0.007 U/ml/min during first 24 hours when disposed engine oil was used as carbon source. Data revealed new and broad band which is related to O-H stretching formed at 3421 cm^{-1}, though new band occurred at 3424 cm^{-1} and 1645 cm^{-1} after bioremediation. While for ethanol production 1gm of DEO contaminated soil was added to 50ml of MSM medium and incubated for 10 days at 40ºC and pH 7. GC-FID analysis revealed that the soil mixed culture was able to produce ethanol from DEO contaminated soil through microbial fermentation. Subsequent Lipase optimization parameters revealed that this bacteria could produce the highest lipase activity 0.161±0.067 U/mL/min when the 4% (v/v) disposed engine oil was used as carbon source. The best nitrogen source was urea it was able to enhance lipase production up to 0.240±0.063 u/ml/min. In addition of surfactant Tween 80 could also enhance lipase production up to 0.221±0.001 U/mL/min. optimal pH value and temperature was 7.0 and 30°C has achieved 0.095±0.002, 0.125±0.013 U/mL/min respectively. Subsequent optimization of parameters for DEO degradation revealed that soil microorganisms able to biodegrade the oil by 69.23% under optimal conditions of pH 7, at 40°C for 10 days of incubation. The best condition preferred by the soil microorganism was under static and aerobic conditions which enhanced the microbial degradation of the crude engine oil. Addition of surfactant Tween 20 could also enhance the hydrocarbon degradation

TABLE OF CONTENT

TITLE PAGE

ABSTRACT **ii**

TABLE OF CONTENT **iii**

LIST OF TABLES **v**

LIST OF FIGURES **vi**

LIST OF ABREVIATIONS **vii**

CHAPTER 1 INTRODUCTION **1**

1.1 Introduction 1

1.2 Problem Statement 2

1.3 Objective of the Study 2

CHAPTER 2 LITERATURE REVIEW **4**

2.1 Introduction 4

2.2 Disposed engine oil (DEO) 4

2.3 Chemical composition of disposed engine oil 4

2.4 Environmental and health impact of disposed engine oil 5

2.5 Microbial degradation of disposed engine oil 9

2.6 Lipase Enzyme 7

2.7 Factors Influencing Biodegradation of Disposed Engine Oil **Error! Bookmark not defined.**

2.8 Potential Useful By-products from Biodegradation of Disposed Engine Oil 16

2.9 Production of Ethanol from Microbial Fermentation of Disposed Engine Oil 18

2.10 Advantages & disadvantages of Biodegradation 19

2.11 Summary 20

CHAPTER 3 METHODOLOGY **22**

3.1 Introduction 22

3.2 Sampling and Sample Processing 12

3.3 Isolation of hydrocarbon degrading bacteria 12

3.4 Screening And Isolation Of Extracellular Lipase Producing Bacteria 26

3.5 Biodegradation Of Disposed Engine Oil For Lipase Production 27
3.6 OFAT Methods Controlling Lipase Enzyme Productivity 29
3.7 Extracellular Lipase Production Media 17
3.8 Deo Contaminated Soil Biodegradation For Ethanol Production 17
3.9 Preliminary Screening for Biodegradation of DEO Contaminated Soil 18
3.11 The Optimum Conditions for Biodegradation of DEO Contaminated Soil for Ethanol Determination 36
3.12 Disposed Engine Oil Biodegradation 36
CHAPTER 4 RESULT AND DISCUSSION 37
4.1 Introduction 37
4.2 Isolation and Purifecation for Haydrocarbon-Degrading Bacteria and The Presence of Soil Mixed Cultture and Protein Content 37
4.3 Isolation Of Lipase-Producing Bacteria 24
4.4 Partial Identification Of Bacterial Isolate Gs-3 Using Gram-Staining 25
4.5 Protein Content Assay 25
4.6 H Nuclear Magnetic Resonance (H-Nmr) Analysis Of Disposed Engine Oil 43
4.7 Analysis Of Recoveredcrude Extract After Biodegradation 44
4.7.1 Fourier Transform Infrared Spectroscopy (FTIR) Analysis 44
4.7.2 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis 46
4.8 OFAT Study Controlling Lipase Production from DEO Contaminated Soil 47
4.8.1 Effect of Disposed Engine Oil Concentration. 48
4.8.2 Effect of Different Nitrogen Sources 49
4.9 Preliminary screening for biodegradation of DEO contaminated soil 56
4.10 OFAT Methods controlling biodegradation of DEO contaminated soil 57
4.11 The Optimum Conditions for Biodegradation of DEO Contaminated Soil 69
4.12 Gas Chromatography - Flame Ionization Detector (GC-FID) Analysis 70
CHAPTER 5 CONCLUSION AND RECOMMENDATION 75
5.1 Introduction 75
5.2 Conclusion 75
REFERENCES 77

LIST OF TABLES

Table 4.1	Screening of Lipase enzyme activity using Qualitative and Quantitative methods of selected bacterial isolates.	41
Table 4.2	The protein content of DEO contaminated soil samples when grown on NB media in replicates	43
Table 4.3	Chemical structure of crude extract produced by GS-3 grown on disposed engine oil as sole carbon sources by GC-MS analysis.	47
Table 4.4	Total hydrocarbon content before and after biodegradation determined using Toluene as the best solvent.	56
Table 4.5	The summary of optimum parameters and conditions required for efficient disposed engine oil degradation.	70
Table 4.6	The chemical components found in the internal standard used for GC-FID Analysis.	72
Table 4.7	The percentage of ethanol produced after 10days of incubation in 50ml degradation medium under optimum condition.	73
Table 4.8	The percentage of ethanol production from the continuous microbial fermentation of disposed engine oil in one liter MSM.	74

LIST OF FIGURES

Figure 2.1	Factors affecting biodegradation of PAH contaminated soils .	14
Figure 3.1	The flowchart of entire research project methodology.	23
Figure 3.2	Collection and sampling of the DEO contaminated soil samples.	24
Figure 4.1	The serial dilution of DEO contaminated soil sample technique.	38
Figure 4.2	Bacterial colonies isolates of oil degrading microorganism grown on the nutrient agar plate with dilution factor (a) 10-1and (b) 10-2.	39
Figure 4.3	(MSM) medium inoculated with 1 mL of soil suspension after 2 days of incubation.	40
Figure 4.4	Screening for extracellular lipase enzyme on olive oil agar. Positive strain produced yellow zone around it.	41
Figure 4.5	Gram-negative GS-3 bacterial isolate under phase contrast field microscope at 100 × magnification.	42
Figure 4.6	H-NMR profile of disposed engine oil	44
Figure 4.7	FTIR analysis profile of control sample	45
Figure 4.8	FTIR analysis profile of degraded engine oil after 14 days of incubation	46
Figure 4.9	GC-MS chromatogram of recovered crude extract	47
Figure 4.10	Extracellular lipase activity of lipase production and protein content by GS-3 over 96 hours of incubation:	48
Figure 4.11	Effect of different disposed engine oil concentrations on lipase production and protein content over 24 hours of incubation period.	49
Figure 4.12	Effect of different nitrogen sources on lipase production and protein content over 24 hours of incubation period.	50
Figure 4.13	Effect of different initial pH-value on lipase and protein content over 96 hours of incubation period.	51
Figure 4.14	Effect of Incubation Temperature on Lipase and protein content from DEO degradation by GS-3 isolate.	52
Figure 4.15	Effect of addition of different surfactants on lipase production and protein content by GS-3 isolate over 24 hours of incubation period	54
Figure 4.16	Effect of ingredient removal from production medium on lipase production and protein content over 24 hours of incubation period	54
Figure 4.17	Three replicates of sample were prepared containing 1g of DEO contaminated soil in 50ml of MSM.	56
Figure 4.18	Shows the effect of incubation period on the hydrocarbon concentration and biodegradation %	58
Figure 4.19	Shows the effect of different pH-value on the biodegradation of hydrocarbon concentration and in DEO % sample.	59

LIST OF ABREVIATIONS

DEO	Disposed Engine Oil
ECEC	Effective Catian Exchange Capacity
FTIR	Fourier Transform Infrared Spectroscopy
GC-FID	Gas Chromatography Flame Ionization Detector
BSA	Bovine Serum Albumim
TBA	Tributyrin Agar
NB	Nutrient Broth
MSM	Mineral Salt Medium
BH	Bushnell Haas
OFAT	One Factor At a Time
SDS	Sodium Dodecyl Sulfate
GS-3	Gambang Shop-3
GS-2	Gambang Shop-2
BSA	Bovine Serum Albumin
S.D.	Standard deviation
DEO	Disposed Engine Oil
GC-FID	Gas chromatography –Flame Ionisation detector
ICP-MS	Inductively Coupled Plasma - Mass Spectrometry
UV-Vis	Ultraviolet Visible

CHAPTER 1

INTRODUCTION

1.1 Introduction

Disposed engine oil (DEO) is the refined products of crude engine oil. It consists of a long chain saturated hydrocarbons which known as base oil additives. Engine oil plays important role in reducing the friction between the parts of automobile engines so that everything run smoothly. The special feature of engine oil or lubricating oil that enable it to be used for automotive use is its viscosity. There are high content of heavy metals and polycyclic aromatic hydrocarbons that could lead to chronic hazards such as mutagenicity and carcinogenicity. Exposure towards high oil concentration for a longer time may cause the development of kidney or liver disease, damage to the bone marrow and higher risk of cancer (Abioye et al., 2010). DEO also renders the environment unsightly and constitutes a potential threat to humans, animals and vegetation.

Biodegradation provides an alternative and efficient way to speed up the clean-up processes as a countermeasure to remediate soils contaminated with oils (Babu et al., 2011). It remains one of the productive ways to reclaim soils polluted with hydrocarbons. The effort is mainly depend on the presence of the petrogenic organisms that have the ability to degrade the broad array of components in the contaminant. Contagion of soil by petroleum hydrocarbons triggers the growth of indigenous microbes which are capable of exploit the crude oil hydrocarbon as their carbon and energy source by that degrading the pollutants. Despite the fact that experiential and essential conditions varied remarkably in each research some typical tendency have

proved that Gram negative bacteria dominate during bioremediation of the crude oil (Onuoha et al., 2011).

The biodegradation method using microorganism fully relies on the enzymatic activities where it secretes extracellular enzymes that help to degrade and utilize the hydrocarbon as sole carbon source and energy. For example, Bacillus pumilus, Arthrobacter sp, and Pseudomonas sp able to secrete extracellular lipase throughout engine oil degradation process. Besides, lipase activity in soil also used as an indicator of oil biodegradation Lipase Enzyme will break down lipid into fatty acid and glycerol (Sharma et al, 2014). Currently, there are many researchers focusing on the utilization of the crude glycerol that obtained as a byproduct of biodiesel production industry into value added products. This is because glycerol is low in cost and has high degree of reduction. However, so far there is no any research review discussed about the transformation of end product from biodegradation into a useful product. Therefore, glycerin obtained from the biodegradation of disposed motor oil can undergo fermentation to produce valuable products such as ethanol, citric acid, 1,3-propanediol, lactic acid, biosurfactant and succinic acid (Mazumdar et al., 2010).

1.2 Problem Statement

Based on the summary of sales and production data of Malaysian Automotive Association, it shows that the total number of registered vehicles in Malaysia increases drastically every year. According to the report, in 2015 there is an increase of 24.17% over the 2009 figure of 536 905 units. The registered vehicles covers all type of vehicle includes tractors, buses and motorcycles (Road Transport Department Malaysia). As the consequence of the increase in the number of vehicles a huge amount of disposed engine oil can be obtained annually. Contamination of soil by oil causes it lose its useful properties such as fertility, water holding capacity, permeability and binding capacity. Used engine oil is a very dangerous polluting agent due to its chemical composition.

Therefore, biodegradation can act as an alternative strategy in order to degrade and bio-convert DEO contaminated soil with the help of different microorganisms into ethanol and DEO into lipase enzyme.

1.3 Objective of the Study

The objectives of this research project are:

a) To utilize and screen for lipase producer among the hydrocarbon degrading bacteria.
b) To optimize the parameters of lipase production using DEO.
c) To characterize the biodegradation products of DEO.

1.4 Scope of the Study

1. This dissertation focuses on production of useful end product such as bioethanol and lipase enzyme using DEO as sole carbon source.

2. Soil microorganism that could degrade disposed engine oil was found and used for entire research. The mixed culture found in the soil sample believed to have higher efficiency to biodegrade the DEO compares to the single colonies. Besides, bacterial isolates were also used for lipase enzyme production in presence of DEO as the sole carbon source.

3. Different factors would be tested and studied to optimize the production of profound lipase activity. Biodegradation of disposed engine oil would be carried using the microbial consortium and the resulting product was extracted for technical analysis for identification of useful metabolites.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter emphasis on the detailed information regarding biodegradation of disposed engine oil. The chemical composition of disposed engine oil was discussed here. Besides, parameters affects biodegradation process would be highlighted as well. The potential useful products that could be produced (ethanol and lipase enzyme) from DEO were briefed in this chapter.

2.2 Disposed engine oil (DEO)

It is the refined product of crude oil Consists of long chain saturated hydrocarbon. High content of polycyclic aromatic hydrocarbon (PAH) and heavy metals such as Mg, Ca, Zn, Pb and S. Act as a lubricant to reduce friction between the parts and enable the engine to runs smoothly.

2.3 Chemical composition of disposed engine oil

Engine oil is purified from core oil and is constitute of long hydrocarbon chain and other organic compounds to magnify its natural properties (Butler and Mason, 1996). It acts as a lubricant to speed up the function of the engine. Disposed engine oil also known as spent oil that is obtained after servicing and subsequent draining from workshop, generators and industrial machines. They are often disposed-off indiscriminately in certain countries. A range of physical and chemical alteration went through by motor oil during the regular engine operation.. Some component of engine oil especially the used engine oil are known as hazardous to the environment and public health. Fresh and used engine oil can be differentiated based on their chemical configuration. According to Kupareva et al., (2012), the nonstop engine modification cause changes in the configuration of new engine oils and new oil additives. It also showed that there is a presence of polycyclic aromatic hydrocarbon in used motor oil and absent in fresh motor oil. There is 98.9% (w/w) of aliphatic hydrocarbons, 0.94% (w/w) aromatic hydrocarbon and 0.08% (w/w) olefinic hydrocarbons in used engine oil. The aromatic hydrocarbon found in used engine oil is 0.67% (w/w) higher than in fresh engine oil due to the process of oxidation during engine operation aromatic hydrocarbons, olefins, water and short chain of hydrocarbons were detected in used

engine oil under nuclear magnetic resonance (NMR) spectroscopy analysis. The spent motor oil contains substantial amount of harmful hydrocarbons and heavy metals. The heavy metals found in engine oil are added in order to improve the lubricating properties and it also act as an effective antioxidant. There are high percentages of new and delicate hydrocarbons in the fresh engine oil. According to Obayori, (2014), fresh motor oil regularly consists of very little amount of polycyclic aromatic hydrocarbons (PAHs). In contrast, used motor oil contain higher percentage of organic compounds such as napthalene, alkyl benzenes, phenol, methyl napthalene and PAHs as a result of pyrosynthesis. Besides that used engine oil also contain few amounts of additives and heavy metals such as lead, zinc, barium and magnesium resulting from engine wear.The organic compounds and heavy metals found in motor oil effluent considerably toxic to the environment and can generate cancer causing agents and endocrine disrupters (Babu et al., 2011). Moreover, (Obini et al., 2013) have discussed that waste engine oil is a concoction of various chemicals comprised of crude oil hydrocarbons, chlorinated biphenyls, additives, heavy metals and decomposition product that originate from part of engine as they abrade. The source, duration of use and degradation product formed in the oil over time or throughout use are the factors that determine the precise configuration of the used motor oil. The polycyclic aromatic hydrocarbon (PAH) present in the used engine oil is belongs to the batch of hazardous organic contaminant which consists of two or more attached benzene aromatic rings. PAH formed due to the partial combustion of organic matters present in the disposed engine oil.

2.4 Environmental and health impact of disposed engine oil

Effects of spent engine oil on soil properties: The results of the soil analysis before and after harvest. There was no significant difference ($P>0.05$) in the soil pH between the control and the contaminated soil. However, pH ranges were between 4.5 and 4.7. Soil pH is a major factor influencing the availability of elements in the soil for plant uptake (Marschner, 2011). Many metal cautions are more soluble and available in the soil solution at low pH (below 5.5) including Cd, Cu, Hg, Ni, Pb, and Zn (McBride, 1994). The retention of metals to soil organic matter is also weaker at low pH, resulting in more available metal in the soil solution for root absorption.

The organic C and N contents of the contaminated soils increased compared to the control. This resulted from application of the spent oil to soil. Crude oil, from which the engine oil is produced, contains principal elements such as oxygen, nitrogen and sulphur other than hydrogen and carbon (Selley, 1998). The oil increased the Mg content of the soil, the degree however decreasing with increasing oil concentrations. P was reduced in the oil contaminated soils compared to the control and this agrees with the findings of (Ogboghodo et al., 2004). The soil texture (sandy soil) was not significantly affected by the SEO. Spent engine oil used in the present study contained 1.3mg/l of Cu and 5.2mg/l of Fe before application to soil. Thus, the oil-contaminated soils contained more heavy metals than the control and the values increased with increasing oil concentration. This implies that soils retain heavy metals in spent engine oil even during drainage. This behavior may result from certain mechanisms such as chelation and sorption by soil. (Yong, 2000) reported that there is a bonding relationship between contaminants and soil surfaces due to sorption forces. The contaminant solutes

in solution become attached to the surface of the soil (solids) particles through mechanisms, which seem to satisfy the forces of attraction from the soil solids (surfaces) (Yong et al., 1992). The heavy metals, being positively charged, are electrostatically attracted to the negative charges on the clay particles (Yong, 2000). Organic complexion of metals may also occur when the solid state humic material binds metals into a ring-typed structure (ligand molecule) most commonly a chelate (Harrison, 1996; Sparks, 2003). In addition, Malaysian Environmental Quality Act and Regulations (101) have the categorized it as a schedule waste. Based on the regulation, only the licensed premises have the permission to collect and store the used engine oils for disposal. Moreover, the illegal disposal of the used motor oils will be punished by law. However, there are still many cases of irresponsible disposal of used motor oil occur. It might be due to the high transportation cost and time consuming which triggers them to dispose the used engine oils improperly by discharge them into the nearby river or dumping at vacant land. Long term exposure towards this highly concentrated harmful used engine oils can cause major health problems to people and some environmental issues. There are many researches that have been discussed about the health risks caused by the exposure towards hazardous disposed engine oils. Kidney failure, liver problems, bone marrow damage and cancer are few of the major health problems encountered by people due to exposure towards these toxic hydrocarbons present in the oil contaminated area. A person with high accretion of heavy metals such as lead, cadmium and aromatic compounds such as PAHs, chlorinated biphenyls may suffer from kidney and liver damage. The PHAs global emission sources and concentration in soil over time according to Kuppusamy et al., (2017). Furthermore, present of these contaminants in the soil which also can be drained into water bodies due to heavy rain can disrupt the balance of ecosystem which eventually lead to greenhouse effect. Soil pollution especially by the spillage of oil had been spotted to cause undesirable growth in plants. Moreover, there are certain significant impacts on plants addressed by few research findings such as plant height deduction, irresponsible disposal on plants, chlorophyll loss and reduction of plant protein level. For example, (Adewole & Aboyeji, 2013) reported that most of the mobile components from spent motor oil could be obstructed by the roots of germinating plants and carried to the wholesome parts of the plant. Therefore this fact strongly indicates that such reaped crops can cause some health consequences for the humans who consume it. Besides, spent motor oil is less viscous than fresh engine oil. Thus when the used engine oil disposed into the land, it tremendously effect the aeration of soil due to the oil which absorbed recalcitrantly by the soil particles. In this case, both the soil and water are contaminated by this disposed used engine oil. This is because heavy rain fall can transfer the spilled engine oil from the soil into the nearby river or sea. Thus, it directly effects and kills the aquatic lives in the water environment. According to Zakaria et al, (2002), the main source of sedimentary PAHs in twenty nine Malaysian riverine is proven to be the used engine oil. The presence of PAH is due to the illegal dumping of used motor oil and the leakage from the repaired vehicles. The balance of aquatic ecosystem is disrupted by the presence of the hydrocarbons in the river and sea.

2.5 Microbial degradation of disposed engine oil

2.5.1 Biodegradation & Bioremediation

Biodegradation, as a ubiquitous secondary alteration in reservoirs, is a principal process that forms most of the heavy crude oils (Head et al., 2003; Oldenburg et al., 2009). Sequential and systematic variations in the chemical compositions of crude oils, such as the preferential removal of hydrocarbons and the relative preservation of non-hydrocarbons, are commonly observed in reservoir biodegradations (Meredith et al., 2000; Koopmans et al., 2002; Huang et al., 2003; Ross et al., 2010; Liao et al., 2012) as well as in laboratory simulations (Palmer,1993; Taylor et al., 2001; Watson et al., 2002; Jones et al., 2008; Erstad et al., 2009). These changes give rise to an increase in oil density, acidity, and viscosity, which produce negative economic consequences. In general, studies on the biodegradation of crude oil, both on the negative and positive sides, are of concern in petroleum production and oil refining, as well as with studies in other fields such as organic geochemistry, geobiology and environmental science. Bioremediation is one of the promising technologies that is available now and has more benefits to deal with hydrocarbon contaminated soils. It involves the use of microorganisms that are capable to degrade hydrocarbons through their enzymatic activity. Microorganism is a natural recycler which transforms the natural and synthetic chemicals into source of carbon and energy to initiate their growth and able to utilize oil as a source of food and many of them can produce formidable surface active compound that can emulsify oil in water and assist its removal. Microbial emulsifier is non-toxic and biodegradable. This technology is more environmental friendly and low in cost. Enzymatic activity of the microorganism can be used as a biochemical and biological indicator to study the hydrocarbon degradation. Due to its clarity and instance, lipase activity can be used as a perfect guide to investigate the decontamination of hydrocarbon in polluted soil (Mahmoud et al., 2015). There are many researches that have been done to monitor the decontamination of hydrocarbon contaminated soil and it was found that lipase enzyme activity acts as one of the major measuring tool test the hydrocarbon degradation. The other biological parameters that also can be used to study the degradation of oil in contaminated soil are microbial respiration in soil, organic carbon content, ATP, dehydrogenase and beta-glucosidase present in soil.

2.6 Lipase Enzyme

Lipase perform essential roles in the digestion, transport and processing of dietary lipids (triglycerides, fats, oils) in most, if not all, living organisms. Genes encoding lipases are even present in certain viruses. (Girod et al., 2002). Most lipases act at a specific position on the glycerol backbone of a lipid substrate . For example, human pancreatic lipase (HPL),(Winkler et al., 1990) which is the main enzyme that breaks down dietary fats in the human digestive system, converts triglyceride substrates found in ingested oils to monoglycerides and two fatty acids. Several other types of lipase activities exist in nature, such as phospholipases (Diaz&Arm, 2003) and sphingomyelinases, (Goñi F, Alonso A, 2002). However these are usually treated separately from "conventional" lipases. Some lipases are expressed and secreted by pathogenic organisms during an infection. In particular, Candida albicans has a large

number of different lipases, possibly reflecting broad-lipolytic activity, which may contribute to the persistence and virulence of C. albicans in human tissue. (Hube et al., 2000). Although a diverse array of genetically distinct lipase enzymes are found in nature; and, they represent several types of protein folds and catalytic mechanisms, most of them are built on an alpha/beta hydrolase fold (Winkler et al., 1990; Schrag & Cygler 1997; Egmond & van Bemmel 1997; Withers-Martinez et al., 1996) and employ a chymotrypsin-like hydrolysis mechanism using a catalytic triad consisting of a serine nucleophile, a histidine base, and an acid residue (usually aspartic acid). (Brady et al., 1990; Lowe ME 1992). Lipases serve important roles in human practices as ancient as yogurt and cheese fermentation. However, lipases are also being exploited as cheap and versatile catalysts to degrade lipids in more modern applications. For instance, a biotechnology company has brought recombinant lipase enzymes to market for use in applications such as baking, laundry detergents and even as biocatalysts (Guo & Xu, 2005) in alternative energy strategies to convert vegetable oil into fuel. (Gupta et al., 2004; Ban et al., 2001) High enzyme activity lipase can replace traditional catalyst in processing biodiesel, as this enzyme replaces chemicals in a process which is otherwise highly energy intensive, (Harding et al.,2008) and can be more environmentally friendly and safe. Industrial application of lipases requires process intensification for continuous processing using tools like continuous flow microreactors at small scale.(Bhangale& Atul, 2012; Kundu et al., 2011) Lipases are generally animal sourced, but can also be sourced microbially. Lipase can also assist in the breakdown of fats into lipids in that undergoing pancreatic enzyme replacement therapy (PERT). It is a key component in Sollpura (Liprotamase).

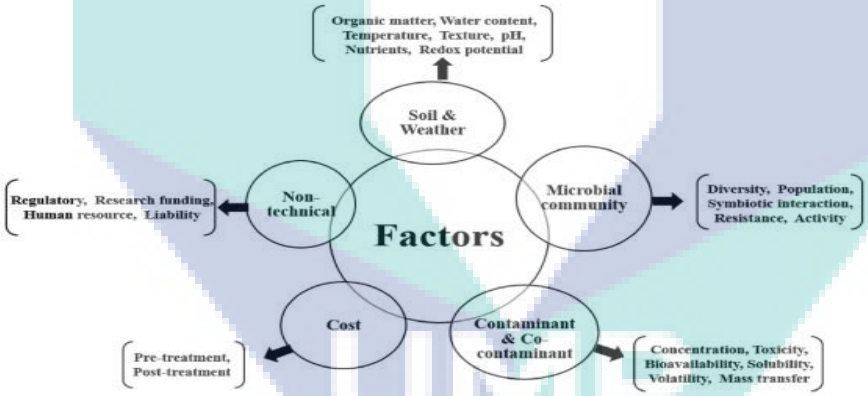

Figure 2.1 Factors affecting biodegradation of PAH contaminated soils

Source: (Kuppusamy et al. 2017).

2.10 Advantages & disadvantages of Biodegradation

This procedure have a lot of advantages as it does not involve excavation of the contaminated soil and hence proves to be cost effective, less expensive than other technologies that are used for cleanup of hazardous waste. There is insignificant site disturbance, so the amount of dirt created is a less significant and synchronized handling of soil and groundwater is possible. It is a conventional process, takes a lesser time, high-class sufficient waste treatment procedure for polluted material such as soil,

and is therefore supposed to be an appropriate waste treatment process. Microbes competent to degrade the contaminant increase in numbers in presence of contaminant and after degradation, the biodegradation population declines. The residues after treatment are normally risk-free products and comprise carbon dioxide, water, and cell biomass. Theoretically, biodegradation is helpful for the total damage of a broad series of contaminants which are lawfully measured to be unsafe and these can be changed to safe products. Biodegradation also requires a very a smaller quantity of effort and can frequently be passed out on site, constantly with no chief disturbance of general actions. As a replacement for transferring contaminants from one environmental medium to a different, for example, from land to water or air, the total damage of target pollutants by means of biodegradation is promising. This technique also eliminates the call for to transfer quantities of waste off site and the probable terror to human health and the environment that can happen for the duration of transport of contaminants. The nutrients added to create microbes grow are fertilizers normally used on lawns and gardens hence not using any hazardous chemicals. Biodegradation changes the damaging chemicals harmless products so the harmful chemicals are completely destroyed (Chaturvedi et al., 2016). Biodegradation, although measured an advantage in the middle of present day environmental situations, it poses some disadvantages, as it is considered challenging because, while additives are added to increase the performance of one particular microorganism, it may be troublemaking to other organisms inhabiting in similar surroundings when done in situ (Vidali, 2001). Microorganisms act well only when the waste materials present permit them to produce nutrients and energy for their growth. The capability of degradation is reduced in unfavorable; in such cases the use of genetically engineered microorganisms is required, even though motivation of native microorganisms is favored. Even if genetically modified microorganisms are released into the surroundings after a definite point of time it is difficult to remove them. Biodegradation is limited to those compounds that are biodegradable, not all compounds are susceptible to rapid and complete degradation. An additional difficulty concerning the use of in situ and ex situ processes is that it is causing more harm than the actual pollution itself as the products of biodegradation may be additional persistent or lethal than the parent compound and also imperfect to those compounds that are eco-friendly.

.

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter describes the methodology that was used in this research to achieve the objectives. This study was carried out the performance of laboratory tests and procedures, testing programs, calibration work and data analysis. First, the appropriate reinforcement method was chosen, followed by the selection and collection of the research materials. In order to investigate the characteristics of the research materials, namely DEO this waste was selected for laboratory test and analysis

Samples of engine oil contaminated soil were collected in sampling bags from mechanic workshops located in Gambang, Kuantan, Pahang, Malaysia. In order to avoid any contamination, the soil must be collected minimum 10cm depth from the soil surface. The soil sample stored in sterile bottles or polythene bags.

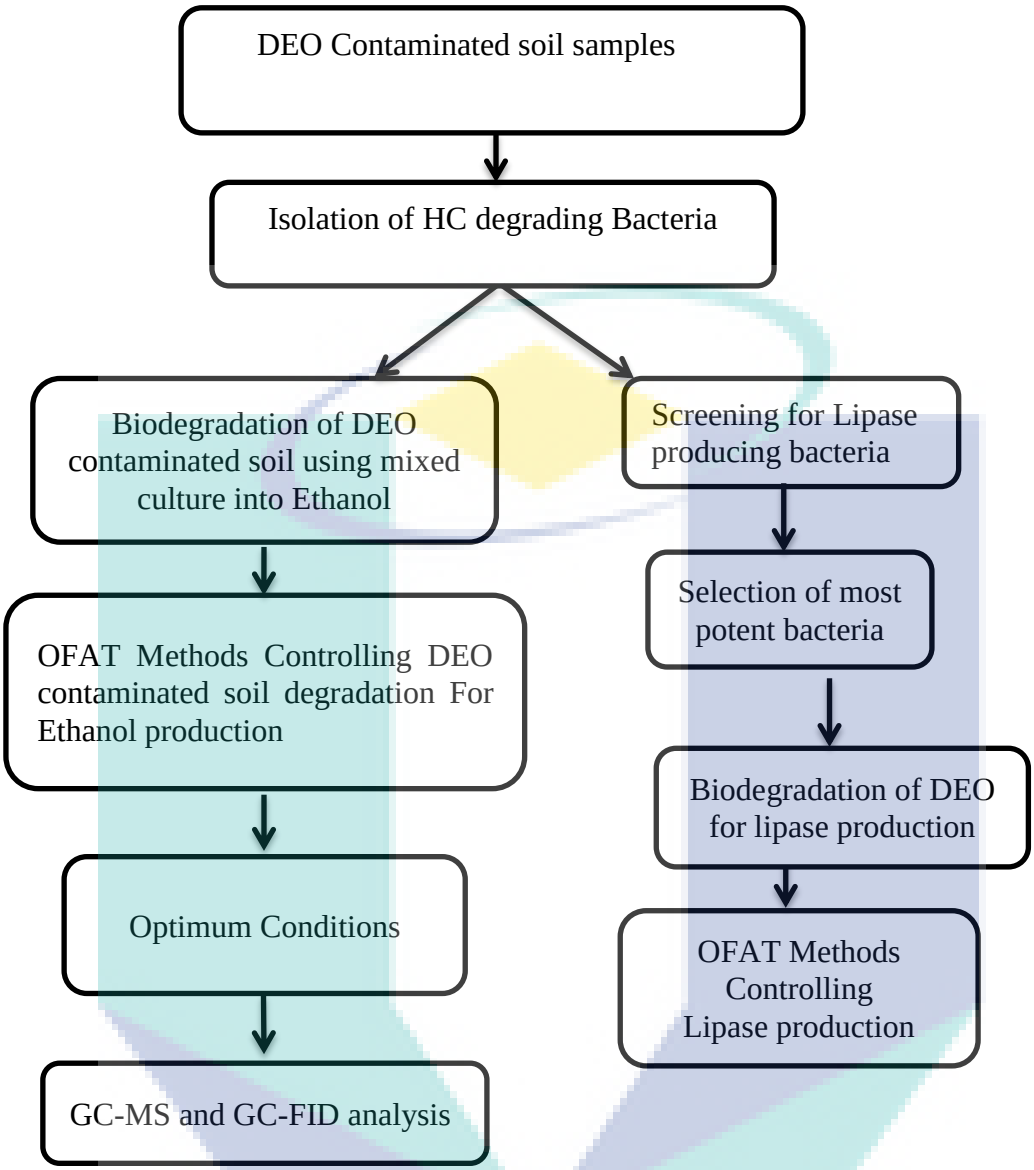

Figure 3.1 The flowchart of entire research project methodology.

3.2 Sampling and Sample Processing

Large particles of soil samples are crushed into small particles using mortar and pestle thoroughly mixed and sieved through a 2mm pore size sieve to remove unwanted large debris. The sieved soil were placed in sterile polyethylene bags, closed tightly and stored at room temperature, thus the soil sample that went through the process of Lipase producer were kept at 4±1ºC (FIig.3.2).

Before Sieving After Sieving

Figure 3.2 Collection and sampling of the DEO contaminated soil samples

3.3 Isolation of hydrocarbon degrading bacteria

Isolation of DEO degrading bacteria was carried out using the method as described by (Adegbola et al., 2014). Two grams of contaminated soil sample was suspended in 9ml of sterilized distilled water and shaken vigorously. Then 1ml of the mixture diluted with 7 dilutions series of each 10^{-1} fold using sterile distilled water. A 100µl aliquot of each series was taken and plated out into Nutrient agar which was prepared according to Appendix A. The plates were incubated at 37°C for 24 to 48 hours. The distinct colonies forming on each plate were observed.

3.3.1 Isolation of Lipase Producers

Bushnell Haas (BH) broth medium was utilized to isolate engine oil-degrading bacteria (Bhattacharya et al., 2015). The medium composition as follows (g/L): $MgSO_4.7H_2O$ (0.2), K_2HPO_4 (1.0), KH_2PO_4 (1.0), $FeCl_3$ (0.05), NH_4NO_3 (1.0), $CaCl_2$ (0.02) with final pH of 7.2 at 25°C. The prepared medium is then sterilized using autoclaved at 121°C for 15 minutes, 50 mL of autoclaved BH broth was prepared in a 250 mL Erlenmeyer flask and supplemented with 2% (v/v) disposed engine oil as sole carbon source bacterial isolates.

3.3.2 Protein Determination

Lowry method (Lowry et al., 1951) was used for protein determination. **S**olution A was prepared by mixing 2.860 g of NaOH with 14.308 g of Na_2CO_3 in 500 ml of distilled water. Solution B was prepared by mixing 1.423 g of $CuSO_4 \cdot 5H_2O$ with 100 ml distilled water. Solution C was prepared by mixing 2.853 g of $KNaC_4H_4O_6 \cdot 4H_2O$ with 100 ml distilled water. Lowry solution consisted of the mixture of solution A, B, and C can only be freshly mixed on the day of the measurement. The mixture of

solution A, B, and C was prepared in ratio of 100: 1: 1. This mixture was then known as solution D. Besides, 1 N Folin reagent was made from 1:1 serial dilution of Folin-Ciocalteu's phenol reagent with distilled water. About 5 ml of solution D was pipetted into a test tube. Then, 0.5 ml of the sample was transferred into the same test tube. The mixture of solution D and sample was mixed and incubated at room temperature for 15 minutes. After incubation, 0.5 ml of Folin reagent was added into the mixture of solution and incubated at room temperature for 30 minutes. Microplate reader was used to measure the absorbance of the sample at wavelength 700 nm. The protein content could be extrapolated from the protein standard curve.

3.3.3 Protein Standard Curve preparation

Bovine serum albumin (BSA) was used as protein standard. The protein standard curve experiment was carried out in dark environment because albumin fraction V is a light sensitive chemical. Different concentrations of BSA were prepared viz: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 mg/mL and the protein determined using Lowry, (1951) Method. The absorbance was measured at Zero nm using microplate reader (Tecan F200-TWT).

3.4 Screening And Isolation Of Extracellular Lipase Producing Bacteria

3.4.1 Preparation of Screening Agar Medium

Two types of screening agar, namely tributyrin agar (TBA) and olive oil agar with phenol red were used to screen extracellular lipase enzyme productivity. Tributyrin agar medium comprised of tributyrin (1% w/v), CaCl2 (0.1% w/v), and agar (2% w/v) was adjusted to pH 7.0 and autoclaved at 121°C for 15 minutes. While olive oil agar plates were prepared by incorporating phenol red (0.01% w/v), olive oil (0.1% v/v), CaCl2 (0.1% w/v), and agar (2% w/v) was adjusted to pH 7.3 and autoclaved at 121°C for 15 minutes. All bacterial isolates were subjected to Gram reaction partial identification.

3.4.2 Qualitative screening method

All isolated oil-degrading bacteria were screened for extracellular lipase activity using qualitative and quantitative assay. Qualitative assay was done by streaking the bacterial isolates on tributyrin agar (TBA) and olive oil with phenol red agar respectively (Lee et al., 2015). Culture plates were incubated at 30°C for 24 hours. According to Sagar et al., (2013), formation of clear halo zone around the colony on TBA indicates extracellular lipase enzyme production. While for the olive oil with phenol red agar, lipase-producing bacteria would turn the red dye into yellow color. These positive colonies for lipase enzyme production were then inoculated at agar slant and subsequently incubated at 30°C for 24 hrs before storing at 4°C as stock culture for further use.

3.4.3 Quantitative screening method

Two loops of 24 hours-incubated petri dish culture was inoculated into 50 mL nutrient broth (NB) supplemented with 2% of disposed engine oil. The culture was incubated for 24 hours at 30°C and 150 rpm in the incubator shaker. Overnight culture was suspended with 0.9% (w/v) NaCl solution to obtain initial cell density of 0.5 McFarland standard. The preparation of Mcfarland Standard could be referred to Appendix A. Submerged microbial culture was incubated in 250 mL Erlenmeyer flasks containing 50 mL of BH broth with 2% disposed engine oil and inoculated with 1 mL of inoculum. After 24 hours of incubation, the culture was centrifuged at 10,000 rpm for 20 minutes at 4°C. Cell free filtrate obtained was used as crude enzyme.

Quantitative assay was carried out by determining lipase activity using titrimetric assay as per (Mustranta 1992). The reaction mixture containing 5 mL olive oil emulsion (Appendix A) and 4 mL of 100 mM pH 7 sodium phosphate buffer (Appendix A). After that, 1 mL of crude enzyme was added into the reaction mixture and incubated at 30°C at 250 rpm for 2 hours. A control was established by using 1 mL of crude enzyme, which is pre-heated at 100°C before added into a reaction mixture. The reaction was stopped by the addition of 5 mL of acetone–ethanol mixture (1:1 v/v) after 2 hours of incubation. The amount of fatty acid liberated was titrated with 0.05 N sodium hydroxide solutions (AppendixA) to a final pH of 10.0. One lipase enzyme unit was defined as the amount of enzyme required for the release of 1 μmol fatty acid per minute under the assay conditions. Enzyme activity was expressed as units (U) per milliliter of enzyme extract and could be calculated using equation (1).

Lipase Unit Activity (U/mL/min) was calculated using the following equation:

$$\frac{\mathrm{N[NaOH]} \times \mathrm{Volume\,of\,NaOH\,titrated} \times 1000}{\mathrm{incubation\,time}} \quad (1)$$

3.5 Biodegradation Of Disposed Engine Oil For Lipase Production

3.5.1 Biodegradation of Disposed Engine Oil

MSM liquid medium was used as the production medium. Fifty milliliter of MSM medium in a 250 mL of Erlenmeyer flask was prepared. The MSM medium was supplemented with 2% (v/v) of disposed engine oil as sole carbon sources for the bacterial isolate. 1 mL of inoculum was transferred into the production medium and incubated at 30°C and 170 rpm on a rotary incubation shaker for 14 days.

3.5.2 Recovery of crude extract for lipase enzyme

The fermentative product was extracted using liquid-liquid extraction technique as described (Adegbola et al., 2014). A 50 mL of trichloromethane was added to broth culture in conical flask and shaking thoroughly. The mixture was then transferred into separating funnel and allowed to stand for two hours. The layer containing the organic

solvent and residual oil is emptied into a beaker. After that, the crude extract was subjected to GC-MS column used: non-polar

3.5.2.1 Charactraization of Extracted DEO by GC-MS

Analysis condition: temperature of injector and detector is maintained at 250 ° C and 350 ° C respectively. Column is set at initial temperature of 70 ° C, this is held for 2 min, then be increased at 10 ° C / min to 320 ° C and held for 10 min. And FTIR analysis for identification of component in the crude extract FTIR spectrometer (A Nicolet 6700 from Thermo Nicolet Corp., Madison, WI) equipped with a deuterated triglycine sulphate (DTGS) as a detector and a KBr/germanium as beam splitter, interfaced to Computer operating under Windows-based, and connected to software of the OMNIC operating system (Version 7.0 Thermo Nicolet), was used during FTIR spectra acquisition. The instrument was maintained with the automatic dehumidifier to diminish water vapor interference. A few drops of each sample were positioned in contact with attenuated total reflectance (ATR) on a multi-bounce plate of crystal at controlled ambient temperature (25 °C).

3.5.2.2 Charactraization of Extracted DEO by FTIR

All FTIR spectra were recorded from 4000 to 650 cm−1, co-adding 32 interferograms at a resolution of 4 cm−1 with strong apodization. These spectra were subtracted against background air spectrum. After every scan, a new reference air background spectrum was taken. The ATR plate was carefully cleaned *in situ* by scrubbing with hexane twice followed by acetone and dried with soft tissue before filling in with the next sample, and made it possible to dry the ATR plate. The plate cleanliness was verified by collecting a background spectrum and compared to the previous one.

3.6 OFAT Methods Controlling Lipase Enzyme Productivity

Different parameters controlling lipase productivity were studied from biodegradation of DEO using most potent bacterial isolates. Parameters are important to determine the factors, conditions and sources that can improve and maximize lipase enzyme productivity. The parameters used were effect of different DEO concentration, nitrogen sources, surfactants, temperature, initial pH-value and ingredient removal from production media.

3.6.1 Disposed Engine Oil Concentration

The effect of different concentrations of DEO as carbon source on lipase production was studied from 1 to 4% whereas other parameters unchanged. The lipase activity and protein content was measured every 24 hours intervals until 96 hours of incubation. It was incubated at 37□C for 96 hours.

3.6.2 Nitrogen Source

Effect of different nitrogen sources on the lipase production was studied by replacing the original nitrogen source, ammonium nitrate with three other nitrogen sources, namely urea, peptone, and ammonium sulfate at concentration of 1% (w/v). Culture using the original nitrogen source was establish and served as control. The lipase activity and extracellular protein content was measured every 24 hours until the end of incubation period. It was incubated at 37ºC for 96 hours.

3.6.3 Addition of Surfactants

The effect of different surfactants on lipase enzyme production was analyzed by using three different surfactants namely Tween 80, Tween 20, and triton X-100.Were tested individually at amount of 50 µL in MSM medium with 2% disposed engine oil. Culture free surfactant was served as control. Other parameters remained unchanged. Lipase activity and protein content was measured every 24 hours until the end of experimental period. It was incubated at 37ºC for 96 hours.

3.6.4 Incubation Temperature

The effect of different incubation temperatures on lipase production was studied in range of 30 to 50°C. The selected bacterial isolates were grown on MSM medium at temperature of 30, 40, and 50°C respectively. The lipase activity and protein content was measured every 24 hours for 96 hours. Other parameters were remained unchanged.

3.6.5 Initial pH-value

The effect of initial incubation pH was performed by varying pH of MSM medium from 6 to 9 using 0.1 M NaOH and 0.1 M HCl whereas other parameters remain unaltered. The lipase activity and protein content was measured every 24 hours interval until the end of experimental period. It was incubated at 37ºC for 96 hours.

3.6.6 Ingredient Removal from Production Medium

The effect of trace element on lipase production was carried out by individually removing trace element, $FeCl_3$, $MgSO_4$, and $CaCl_2$ from the production media. Control was MSM medium included all the ingredient. Other parameter remains unchanged. The lipase activity and extracellular protein content was measured every 24 hours until the end of experimental period. It was incubated at 37ºC for 96 hours.

3.7 Extracellular Lipase Production Media

50 mL of menial salt medium (MSM) liquid medium supplemented with 2% (v/v) disposed engine oil was used as production media. Inoculum was prepared by suspending overnight culture in MSM medium to obtain an initial cell density at turbidity of 0.5 Mcfarland standards. A 1 mL of inoculum was used for this section. Submerged microbial culture was incubated at 30°C on a rotary shaker with 170 rpm. The cell free filtrate was obtained every 24 hours and used as the source of crude extracellular lipase enzyme. Cell free filtrate could be obtained by centrifuging the culture broth at 10,000 rpm for 20 minutes at 4°C. The lipase activity in the cell free filtrate was determined by titrimetric assay as previously discussed. The extracellular protein content was determined as per Lowry et al., 1951.

3.8 Deo Contaminated Soil Biodegradation For Ethanol Production

Solvent extraction method (Adegbola et al., 2014) was used to screen for best solvent that able to extract high amount of hydrocarbon from the engine oil contaminated soil sample. Five different solvents namely acetone, hexane, ethanol, methanol, toluene (99%) with a ratio of (1:1) were selected based on their capability to extract hydrocarbons from the contaminated soil as stated in several studies. On gram of the spent engine oil contaminated soil was added to 50ml of each solvent respectively. Then the mixtures were kept in the shaker at 100rpm for 10 to 15 minutes until they form a layer. The organic layer of each mixture was filtrated using nylon syringe filter to avoid the soil particles and transferred into the quartz to read for the absorbance under ultraviolet-visible (UV) 1800 UV-VIS spectrophotometer. The wavelength was adjusted for each solvent accordingly (Toluene 286, Acetone 330, Ethanol 210, N-hexane 200, Methanol 205) nm.

The screening was further preceded using the solvent with the highest absorbance reading as the best solvent for the detection of hydrocarbon level in the oil contaminated soil. Thus, a hydrocarbon standard curve was prepared using the selected solvent in order to obtain the concentration of hydrocarbon in the disposed engine oil contaminated soil based on the absorbance values.

3.8.1 Estimation of Hydrocarbon Using Solid-Liquid Extraction

The concentrated hydrocarbon found in the DEO contaminated soil was obtained using extraction method as applied in (Wang et al., 2007). Eight grams of the contaminated soil sample were mixed with 200ml of the best hydrocarbon extracting solvent selected from the previous screening step and added into the separating funnel. The mixture was shaken for 25minutes and left to be settled for 10 minutes. The top portion containing the solvent along with hydrocarbon was separated from the bottom soil extract. The solvent containing hydrocarbons was then transferred into rotary evaporator (DL10-300 CFC-free Recyclable chiller) to obtain the concentrated hydrocarbon. The boiling point was adjusted according the solvent used. At the end of evaporation process, there was a color changes that can be observed in the hydrocarbon sample. The absorbance reading was recorded for the pure hydrocarbon sample under

the UV-spectrophotometry and remaining sample was stored in the chiller for further use.

3.8.2 Preparation of Total Hydrocarbon Content (THC) Calibration Curve

By using the known concentration of the hydrocarbon sample extracted earlier the total hydrocarbon standard curve was prepared. Different concentrations of hydrocarbon were prepared from range of 0.0 to 0.25µl/ml using solvent dilution and the absorbance was measured using UV-Vis spectrophotometer. Accordingly, the relationship between the hydrocarbon concentration and the absorbance was as follows (equation 2):

$$Y = m X \quad (2)$$

Where, Y is the measured absorbance of sample, X is the concentration of hydrocarbon in the sample and m represents the slope.

The percentage of degradation of hydrocarbon was determined as the difference between the initial and final THC concentrations as stated in (Ibrahim 2016):

$$\text{Degradation (\%)} = [\ (O_i - O_r)\ /\ O_i\ ] \times 100\% \quad (3)$$

Where O_i is the initial hydrocarbon concentration (µl/ml) and O_r is the residual concentration.

3.9 Preliminary Screening for Biodegradation of DEO Contaminated Soil

Mineral Salt Medium (MSM) was utilized for the biodegradation of disposed engine oil contaminated soil (Adegbola et al., 2014). MSM act as the nutrient source and enhance the growth of the oil degrading microorganisms present in the contaminated soil. MSM was prepared using the following salts (g/L): KH_2PO_4 (2.0), $NaNO_3$ (2.0), NaCl (0.8), KCl (0.8), $Na_2HPO_4.12H_2O$ (2.0), $MgSO_4$ (0.2) and $FeSO_4.7H_2O$ (0.001). One liter of MSM was prepared and dissolved in one liter of distilled water with final pH of 7.0. The prepared medium was autoclaved at 121°C for 15minutes. 50ml of the autoclaved MSM was transferred and distributed into sterile four different 250ml conical flask respectively. One gram of disposed engine oil contaminated soil was aseptically inoculated into each conical flask containing MSM. One of the flasks containing the sample used as a control (containing MSM and DEO excluded the microorganism and kept under the same condition) and the rest were triplicates of the samples. All flasks were incubated at 37°C at 170rpm for 4 days.

By using the solvent extraction method the amount of hydrocarbon present in the disposed engine oil contaminated soil before and after biodegradation incubation period was determined. The control containing contaminated soil sample was extracted using the previously selected solvent with the mixing ratio of 1:1, where 50ml of the solvent was added to 50ml of the control and kept in shaker for 15 minutes. After 15minutes, the formation of two layers was observed and the upper layer containing the solvent and residual oil was collected and read for absorbance under UV-Vis

spectrophotometer. The solvent was used as the blank for the absorbance reading. The reading obtained for the control represents the hydrocarbon content in oil contaminated soil before biodegradation. The same steps were repeated for the samples kept in the incubator for 4 days to determine the hydrocarbon present in the waste engine oil contaminated soil after biodegradation. The microbial degradation of disposed engine oil contaminated soil was determined based on the results obtained.

3.10 Biodegradation of DEO for ethanol production using one factor at a time (OFAT) method

Several parameters that have been used in this research in order to enhance the biodegradation of DEO contaminated soil. All the factors were prepared using triplicate of samples. OFAT refers to the approach when determining a factor controlling and other parameters were remains unchanged. It was applied in the following parameters analysis method for biodegradation of DEO. The total hydrocarbon in the sample was estimated using the solvent extraction technique mentioned in previous steps and the absorbance reading obtained was used to determine the concentration of hydrocarbon from the THC calibration curve that has been plotted earlier. Based on the concentration, the percentage of biodegradation of DEO was calculated.

3.10.1 Incubation period

The effect of different incubation periods on the biodegradation of DEO contaminated soil was studied over the range of 0 to 10 days. A 2gram of oil contaminated soil was inoculated into 50ml of MSM medium and incubated at 37°C for 0, 2 ,4, 6, 8 and 10 (day) respectively. The pH of the medium was maintained at 7.0 and the samples were kept in static and aerobic conditions. The total hydrocarbon was determined using liquid-liquid extraction technique as previously mentioned.

3.10.2 Initial pH_value

The effect of different initial incubation pH values on biodegradation of DEO contaminated soil was performed at range of 5, 6, 7, 8, and pH 9. The initial pH was altered using 0.1M NaOH and 0.1M HCl after the addition of the soil sample whereas other parameters remain constant. The total hydrocarbon was measured after 10 days of incubation period.

3.10.3 Incubation Temperature

The effect of different incubation temperatures on DEO was studied in range of 20 to 50°C. The oil contaminated soil mixed culture was inoculated and grown on MSM at temperatures of 20, 30, 40, and 50°C respectively and incubated as previously mentioned. The percentage of DEO degradation was determined after 10 days.

3.10.4 Inoculum Size

Different inoculum sizes were examined to determine their effect on biodegradation of DEO. The amount of inoculum was varying from 0, 1, 2, 3 and 4g which was added to MSM medium and incubated for 10 days. The inoculum size refers to the amount of disposed engine oil contaminated soil per gram. At the end of incubation period the total hydrocarbon content was determined using the same method as mentioned earlier.

3.10.5 Incubation Condition

Effect of aeration on biodegradation of DEO contaminated soil was studied under static and shaking conditions. Replicates containing the soil sample mixed with 50ml of MSM were prepared for each static and shaking condition respectively. Shaker incubator was used at 150 rpm. The flasks were incubated at 37°C for 10 days.

3.10.6 Oxygen Availability

The effect of presence or absence of oxygen on the biodegradation of the oil contaminated soil was examined. The prepared mixtures of contaminated soil and MSM was kept in the normal incubator at 37°C for the aerobic condition and for the anaerobic condition it was kept in anaerobic jar and incubated at 37°. The other parameters were kept constant and the total hydrocarbon was determined after 10 days using Liquid-Liquid extraction technique.

3.10.7 Flask Volume Capacity

Varies sizes of conical flasks were used to study the outcome of flask volume capacities on biodegradation of DEO contaminated soil. A 50ml of MSM was inoculated with 2g of oil contaminated soil was prepared in different conical flasks volumes ranging from 50, 100, 250 and 500ml and incubated for 10 days at 37°C. Other parameters were remained constant.

3.10.8 Effect of Surfactant

Different types of surfactant were examined to determine their effect on the microbial degradation of DEO contaminated soil namely, Tween 20, Tween 80, Sodium Dodecyl Sulfate (SDS) and Triton X-100. A 50ml of MSM medium together with 2g of contaminated soil was prepared in separated flask and each flask was added with 10µl of the surfactant under study respectively. One flask without the addition of surfactant acted as the control. It was incubated at 37°C for 10 days. At the end of incubation period total hydrocarbon was determined using Liquid-Liquid extraction technique.

3.11 The Optimum Conditions for Biodegradation of DEO Contaminated Soil for Ethanol Determination

Based on the findings from the parameters, the optimum condition for each factor was identified and summarized. The biodegradation of DEO contaminated soil under the optimal conditions was examined by preparing 50ml of sample in three replicates and run the sample under the selected ideal conditions. Solvent extraction strategy was used to find the percentage of hydrocarbon degraded from the sample. The aqueous layer obtained was send to GC-FID Agilent Technologies 7890A GC Systems coupled with FID detector analysis FIST UMP,Gambang,Kuantan,Malysia to detect the presence of ethanol in the sample. The absorbance reading was taken for hydrocarbon determination in the residual oil layer obtained from the samples.

3.12 DEO Biodegradation

In order to scale up the microbial degradation of DEO contaminated soil a bioreactor containing one liter of MSM was prepared. All the factors such as pH, surfactant, incubation period, temperature, aeration, presence of oxygen was fixed according to the optimal conditions. One of sample from the previous replicates that had been prepared for optimization of the parameters was used as the inoculum for the 1 liter degradation medium. The bioreactor was operated manually and the optimal conditions were observed every 24 hours until the end of incubation fermentation period.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Introduction

This chapter discusses the properties of DEO and DWF used in this research. Graphs, figures and also tables were used to represent the results obtained from the laboratory tests. The data from the various types of samples were gathered and compared in order to obtain the best results among the samples.

4.2 Isolation and Purefecation for Haydrocarbon-Degrading

Serial dilution and spreading plate technique were used to determine the presence of oil degrading microorganisms in the DEO contaminated soil. This is due to the fact that only those microbes which have the potential to biodegrade the disposed engine oil and use it as sole carbon source can survive and grow in such environment. Besides, in order to prevent the samples from contaminated by the microbes that might present in the laboratory environment all the procedure were carried out aseptically in the laminar flow under sterile condition.

The apparatus and distilled water used for the serial dilution and the nutrient agar preparation were sterilized. According to figures (4.1) and (4.2) showed the presence of bacterial colonies in the nutrient agar plates after 48 hours of incubation at 37°C. Bacteria can grow tremendously fast when provided with sufficient nutrients. Different types of bacteria produced different colonies shapes, some colonies may be colored, some were circular in shape, and others irregular. Each distinct circular

colony represents an individual bacterial colony that has divided repeatedly. Being kept in the incubator for 48 hours at optimum condition, the resulting cells have accumulated to form a visible patch. Most bacterial colonies appear white, cream, or yellow in color, and fairly circular in shape. Besides, presence of fungi colonies also found in the agar plates, but only bacterial colonies were selected in this study. The large and round shaped colonies showed the presence of yeast, fungi which strongly support the facts stated in previous researches that fungi also have the ability to biodegrade disposed engine or petroleum found in the soil (Abioye et al., 2012; Thenmozhi et al., 2013).

The purification technique for isolation of single colony of bacterial isolates was not carried out in ethanol production par from biodegradation of DEO, because we used soil suspension inoculum as a mixed culture. While in case of lipase enzyme production part all isolates were successfully purified using streaking technique.

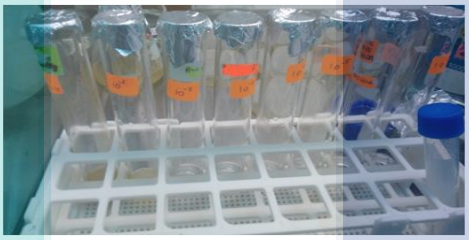

Figure 4.1 The serial dilution of DEO contaminated soil sample technique.

Flasks containing MSM medium with soil mixture was visibly turbid than control after 2 days of incubation, indicating proliferation of engine oil-degrading microorganism (Figure 4.3). Culture broth was plated on BH agar plate and nutrient agar supplemented with disposed engine oil respectively after 7 days of incubation period. The bacterial colonies on nutrient agar were more observable than that on BH agar. Besides, the size of colony on nutrient agar was bigger and morphology of bacteria could be observed.

Hydrocarbon-degrading bacteria with different morphological characteristics were further plated out in order to obtain pure bacterial isolates. A total number of 10 disposed engine oil-degrading bacteria were successfully isolated and purified from soils of different locations were named as strain GS-1 to GS-10 bacterial isolates.

Figure 4.3 (MSM) medium inoculated with 1 mL of soil suspension after 2 days of incubation.

4.3 Screening And Isolation of Lipase-Producing Bacteria

All isolated hydrocarbon-degrading bacterial strains were plated on tributyrin agar and olive oil agar respectively to screen extracellular lipase enzyme. Only 2 out of 10 isolated hydrocarbon-degrading bacteria produced extracellular lipase enzyme were GS-2 and GS-3 isolates respectively. Qualitative screening method showed that both isolates had same lipase activities. However, titrimetric method assay showed that GS-3 isolate exhibited higher lipase activity than GS-2 (Table 4.1).

Table 4.1

Screening of Lipase enzyme activity using Qualitative and Quantitative methods of selected bacterial isolates

Screening Media used		Lipase activity diameter (mm)	
		GS-2	GS-3
Qualitative	Tributyrin agar	5±0.000	5±0.000
	Olive oil with phenol red agar	5±0.000	5±0.000
Incubation time (hr)		Lipase activity of bacterial isolates (U/ml/min)	
		GS-2	GS-3
Quantitative	24	0.058 ± 0.010	0.097±0.007
	48	0.046±0.007	0.074±0.007

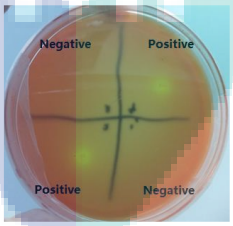

Figure 4.4 Screening for extracellular lipase enzyme on olive oil agar. Positive strain produced yellow zone around it.

4.4 Partial Identification Of Bacterial Isolate Gs-3 Using Gram-Staining

Gram-staining revealed that chosen bacterial isolate GS-3 belongs to Gram negative and rod shape bacteria. This is because GS-3 was not able to retain the crystal-violet and appeared to be red color after safranin staining (Tiwari et al., 2008).

4.5 Protein Content Assay

Protein standard curve that plotted to determine the concentration of protein in the mixed culture from oil contaminated soil samples that prepared in triplicates. Lowry protein assay protocol was carried out in the dark environment and the absorbance at 700nm was determined using micro plate reader.

The protein content of DEO contaminated soil sample was determined using Lowry method and the results were obtained as shown in Table 4.2. The protein content analysis was carried out after 18 hours of incubation in replicates which containing 50ml nutrient broth (NB) inoculated with one gram of oil contaminated soil sample. The fresh uninoculated NB was used as control. Based on the results, it was proven that both control and samples contain specific amount of protein. This is due to the fact the control was already consists of peptone which is a soluble protein necessary for the growth of the microorganisms. However the protein content in the samples was higher than control. This is because the microorganisms able to grow rapidly in presence of DEO sample. An average protein content of 1.407mg/ml was found in the sample containing mixed culture of soil microorganism. Thus, the screening for the presence of oil degrading microorganism and their protein content from the DEO contaminated soil sample have given positive outcomes which become a turning point to proceed the research under study.

Table 4.2

The protein content of DEO contaminated soil samples when grown on NB media in replicates

Incubation time	**Ab at 700nm***	**Protein Content (mg/ml)***
Control	0.462 ± 0.016	0.764 ± 0.010
1	0.819 ± 0.017	1.390 ± 0.013
2	0.827 ± 0.012	1.405 ± 0.008
3	0.839 ± 0.015	1.426 ± 0.015

*The results are expressed in means ± S.D.

4.6 H Nuclear Magnetic Resonance (H-NMR) Analysis Of Disposed Engine Oil

Referring Figure 4.6, intense peak could be observed at 1.2581 ppm and 0.8687 ppm. Formation of these peaks indicated the presence of alkane. Besides, small peak appeared at 7.2458 , 7.1158 and 6.9110 ppm indicated the presence of aromatic rings. Figure 4.26 showed, 1H- NMR analysis revealed that disposed engine oil is mainly composed of alkane. Presence of aromatic rings might be attributed to the formation of Polyaromatic hydrocarbon (PAH) during engine operation. (Dominguez-Rosado and Pichtel. 2003)

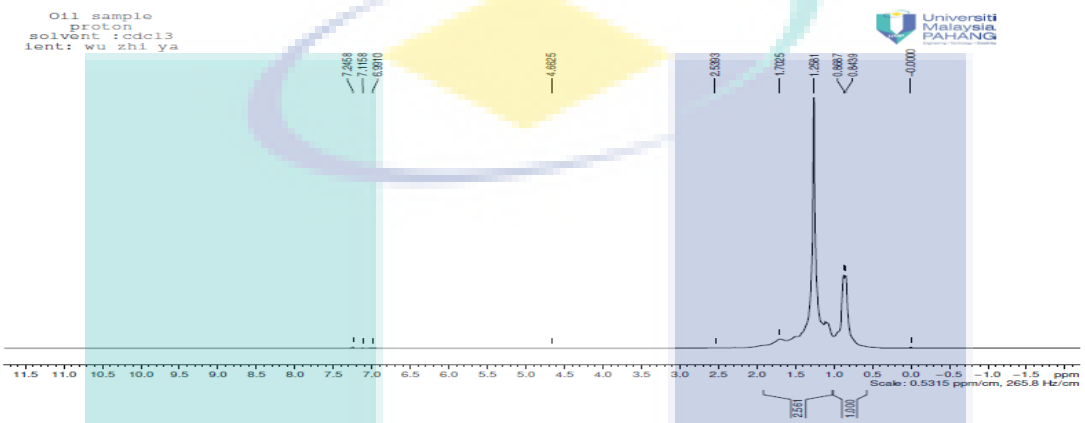

Figure 4.6 H-NMR profile of disposed engine oil

4.7 Analysis Of Recoveredcrude Extract After Biodegradation

4.7.1 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Referring to Figure 4.7 and Figure 4.8, new and broad band which is related to O-H stretching formed at 3421 cm-1. Besides, new band could also be observed at 1706 cm-1 and 1649 cm-1, which is associated Occurrence of these bands signified the formation of carboxylic acid after 2 weeks of biodegradation. FTIR analysis showed that new and broad band which is related to O-H stretching formed at 3421 cm-1 after 14 days of degradation by bacterial isolate GS-3. At the same time, new and short band which is associated carbonyl group could also be observed at 1706 cm-1.Occurrence of these bands signified the formation of carboxylic acid after 2 weeks of biodegradation. This result is corresponding to that of Dominguez-Rosado and Pichtel (2003), who remediated disposed engine oil utilizing phytoremediation technique and remarked that carboxylic acid is one of the intermediates which were produced along the biodegradation process. Today, the application of Fourier transform infrared (FTIR) spectroscopy has increased in food studies, and particularly has become a powerful analytical tool in the study of edible oils and fats (Guillen & Cabo, 2000). The power of FTIR as a quantitative tool lies in its ability to readily carry out the multi-component analyses (van de Voort, 1992). There have been several studies concerning with the characterization, classification, and authentication of edible fats and oils using infrared spectroscopy. Combined with chemometric methods, infrared (IR) spectroscopy is an emerging analytical technique to verify the authenticity of edible oils and fats, due to its

simplicity, rapidity, and ease of sample preparation (Yang, Irudayaraj, & Paradkar, 2005).

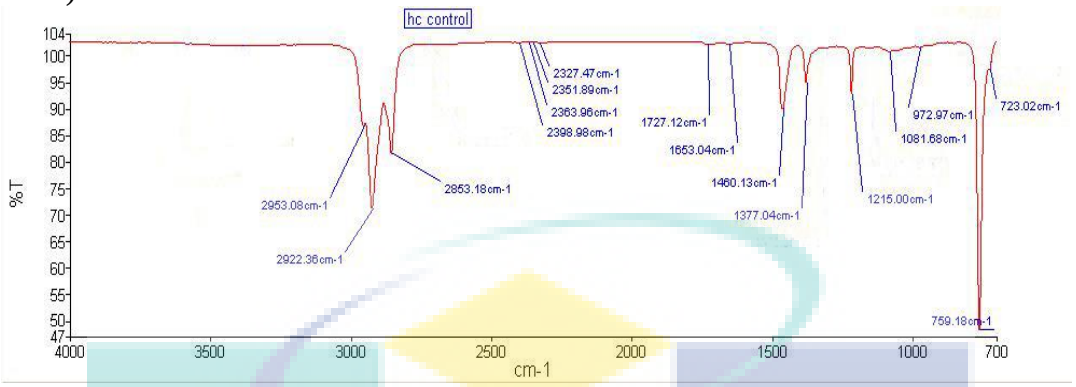

Figure 4.7 FTIR analysis profile of control sample

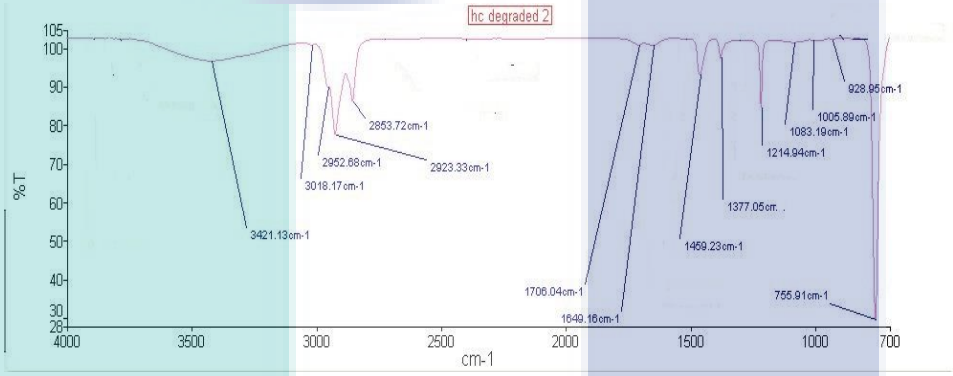

Figure 4.8 FTIR analysis profile of degraded engine oil after 14 days of incubation.

4.7.2 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analysis revealed that crude extract recovered from disposed engine oil after biodegradation contained 3 major compounds: hydrocarbon, alcohol, and carboxylic acid (Table 4.3). The major component formed is organic acid named methyl 3, 4, 5-trimethoxy-2, 6-dinitrobenzoate. Subsequent GC-MS analysis confirmed the presence of carboxylic acid in the degradative engine oil. Referring Table 4.11, GC-MS analysis showed that extracted product contains high percentage of methyl 3,4,5-trimethoxy-2,6-dinitrobenzoate, small amount of complex alcohol, and aliphatic hydrocarbon of nonadecane. Unfortunately, these compounds do not have any industrial application. Besides, Sadouk et al. (2009) also reported that carboxylic acids, which are hexadecanoic acid and octadecanoic acid were the major component formed during biodegradation of diesel oil.

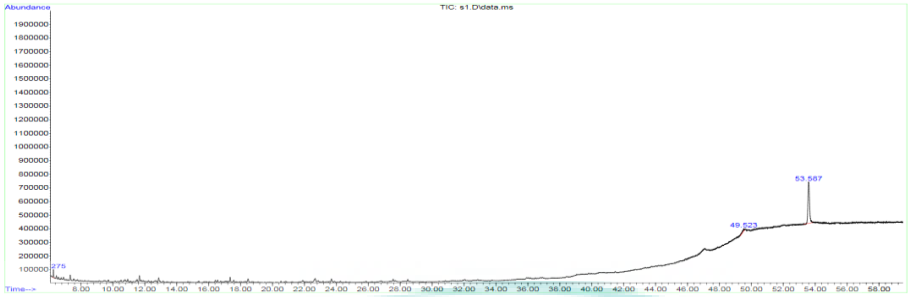

Figure 4.9 GC-MS chromatogram of recovered crude extract

Table 4.3

Chemical structure of crude extract produced by GS-3 grown on disposed engine oil as sole carbon sources by GC-MS analysis

Peak	Retention time (minute)	Name of compound	Area (%)
1	6	Nonadecane	6.11
2	49.5	3,3,7,11-Tetramethyltricyclo-[5.4.0.0 (4,11)]-undecan-1-ol	4.88
3	53.6	Benzoic acid, 3,4,5-trimethyoxy-2,6-dinitro- methyl ester	89.00

4.8 OFAT Study Controlling Lipase Production from DEO Contaminated Soil

Extracellular lipase production by GS-3 over 96 hours of incubation: According to data represented in figure (4.10) bacterial isolate GS-3 was chosen over GS-2 for extracellular lipase enzyme production because it had higher lipase activity, achieving 0.097 ± 0.007 U/mL/min on first day. Besides, the extracellular protein content also showed the similar result of the highest protein content OF 0.4651±0.010 after 24 hours of incubation. However, the protein content did not precisely reflect the total extracellular lipase content because other enzyme would also be secreted along the degradation process of DEO. Adam et al. (2014) reported that oxidase, catalase, lipase, and peroxide were the common extracellular enzyme which would be excreted during biodegradation of DEO. (Appendix B).

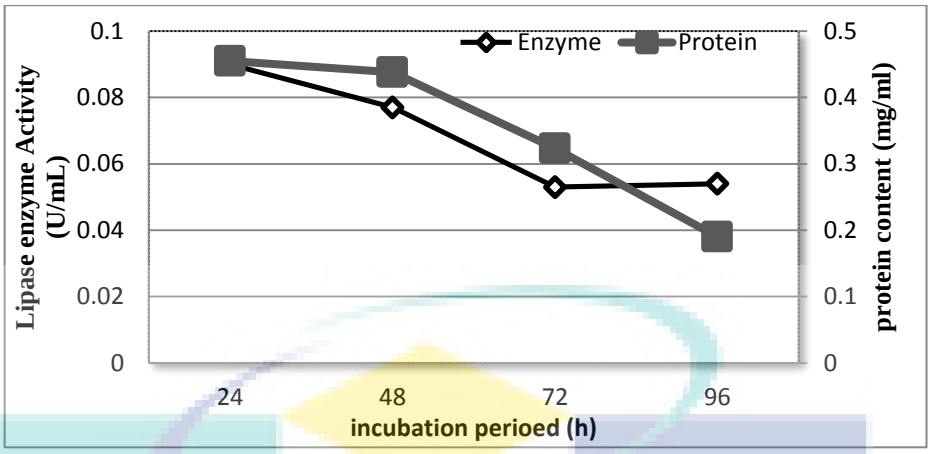

Figure 4.10 Extracellular lipase activities of lipase production and protein content by GS-3 over 96 hours of incubation:

4.8.1 Effect of Disposed Engine Oil Concentration.

Data represented in Figure (4.11) illustrated that lipase activity of GS-3 bacterial isolate was highest when 4 % (v/v) DEO was used as carbon source, with in the first 24 hours of incubation. The protein content was also measured to be 0.850±0.037 (mg/ml). The effect of different concentrations of disposed engine oil as carbon source on lipase activity was studied in this research and revealed that GS-3 isolate was able to produce extracellular lipase enzyme in range of 1 until 4% of disposed engine oil, suggesting no inhibition effect of disposed engine oil on enzyme production within this range. During first 24th hour, lipase activity was highest at 4% recording 0.161±0.067 U/mL/min, followed by 3 and 2%, whereas 1% trailed the most, registered only 0.079±0.003 U/mL/min of lipase activity. This data supported the theoretical statement of (Margesin et al. 2002), who confirmed that lipase activity could be induced and was favored at high petroleum concentration. Amount of lipase activity is roughly similar across 4 different concentrations at other incubation period. The result also showed that GS-3 isolates exhibited highest lipase activity at 72 hours of incubation when the culture medium is supplied with 1%, 2% and 3% DEO, except for 4% of DEO, which gave the highest lipase activity during first 24 hours of incubation. (Appendix B).

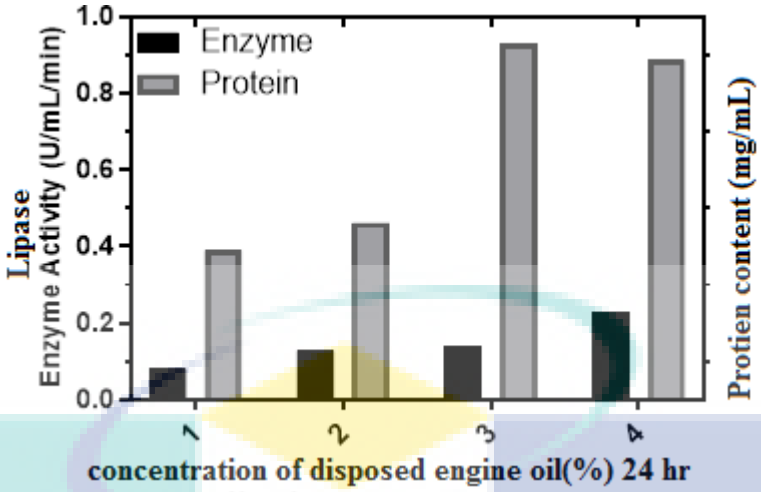

Figure 4.11 Effect of different disposed engine oil concentrations on lipase production and protein content over 24 hours of incubation period

4.8.2 Effect of Different Nitrogen Sources

Data represented in figuer (4.12) showed that urea was the best nitrogen source for extracellular lipase production during first 24 hours of incubation, achieving lipase activity of 0.240±0.063 U/mL/min and protein content up to 0.722±0.001 mg/ml. While peptone was a poor nitrogen source for lipase production of GS-3 isolate as the lipase activity was extremely low, recorded only 0.010±0.001 U/mL/min on first day. Approximately 1-fold higher than control sample which used ammonium sulfate as nitrogen source. Although ammonium sulfates as nitrogen sources could not produce high lipase activity on first day. (Appendix B), on the other hand, peptone was found out to be poor nitrogen sources for GS-3 isolate for lipase production as it was only able to give lipase activity as low as 0.010±0.001 U/mL/min and protein content up to 0.764±0.004 U/mL/min on the first day of incubation period. Therefore, this study showed that inorganic nitrogen sources were better than organic nitrogen sources for lipase production by GS-3. In contrast (Sujatha and Dhandayuthapani 2013) reported that urea inhibited lipase production at concentration above 0.5 mg/L as it was toxic to the culture as high concentration of ammonia produced during urea degradation is toxic to bacteria. This might as well explain the drastic decrease of GS-3 isolate's lipase activity over 4 days when urea was used as nitrogen source. The current also contradicted to other researches which reported that organic nitrogen source was generally better than inorganic source. Gupta et al. (2004) reported that organic sources were better than inorganic sources because ammonium salt creates acidic condition due to liberation of free acid after the utilization of ammonium ions, which might eventually inhibit or interfere the lipase secretion. However, the choice of nitrogen sources for optimal enzyme activity was various among microorganism (Burkert et al., 2004).

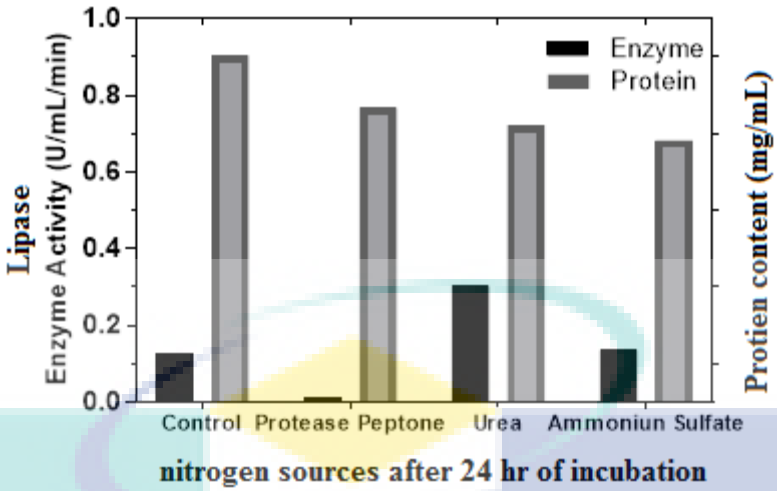

Figure 4.12 Effect of different nitrogen sources on lipase production and protein content over 24 hours of incubation period.

4.8.3 Effect of Initial pH-value on Lipase production

Like temperature, pH was also affecting the metabolic activities of a microorganism. Data represented in Figure (4.13) showed the lipase activity of GS-3 isolate over different range of initial pH. Result showed that pH 7 was the optimal initial pH for lipase production achieving 0.095±0.002 U/mL/min and protein content up to 0.665±0.086 mg/ml after 24 hours of incubation. (Appendix B).

This suggested that GS-3 is a neutrophilic microorganism. When GS-3 was incubated in production medium of initial pH 9, the main problem of using DEO as carbon source was the insolubility of engine oil, compare with pH 8 and 6 has given 0.064±0.005 and 0.044±0.003 with protein content 0.580±0.008 and 0.417±0.014 with the first 24 hours of incubation period.

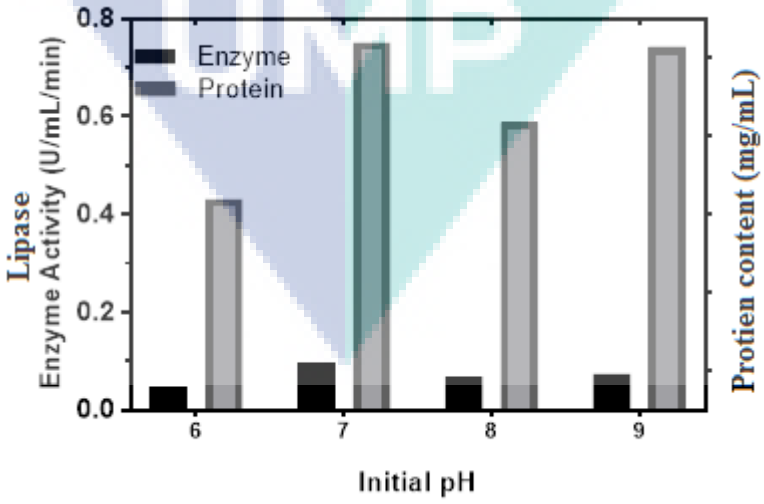

Figure 4.13 Effect of different initial pH-value on lipase and protein content over 96 hours of incubation period

4.8.4 Effect of Incubation Temperature

Data represented in Figure (4.14) showed that after 24 hours of incubation, the lipase activity reached the highest yield of 0.125±0.013 U/ml/min at 30ºC of incubation temperature while lowest enzyme activity observed at 50ºC, recorded as 0.051±0.007 U/mL/min during the same period with protein content up to 0.580 ± 0.010 mg/ml. (Appendix B).

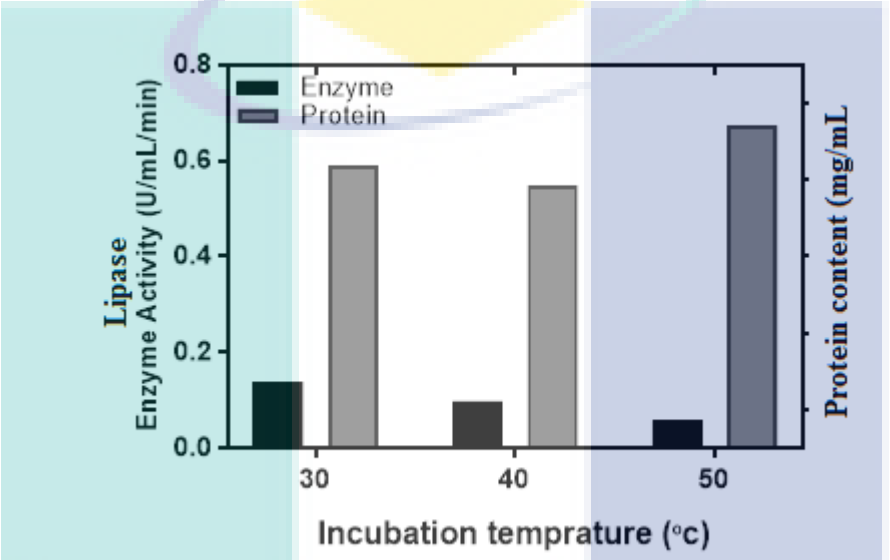

Figure 4.14 Effect of Incubation Temperature on Lipase and protein content from DEO degradation by GS-3 isolate.

Data also showed that GS-3 isolate was mesophilic microorganism. Optimal temperature favoring metabolic and enzymatic activities throughout incubation period, optimal cultivation temperature varies among microorganism. However, 30°C was found out to be the optimal temperature for lipase enzyme production of many other microorganisms. For example, 30°C was the best temperature for *Bacillus* sp. MPTK 912 for production of lipase enzyme from oil mill effluent. also found out that *Pseudomonas* sp. has highest extracellular lipase activity at the same temperature when olive oil was used as carbon sources according to (Rejitha et al. 2012,Narasimha et al. 2011).

4.8.5 Effect of Different Surfactants

Figure 4.15 represented the lipase activity was achieved with addition of different surfactants. Tween 80 could enhance the lipase activity to the highest within 24 hours of incubation, reporting 0.221±0.001 U/mL/min with protein content was also measured and recorded up to 1.907± 0.031 mg/ml. (Appendix B).

Engine oil is hydrophobic in nature and is not miscible with water. Addition of surfactant into the production medium would improve the solubility of DEO, which in turn, increases the bioavailability of substrate to bacteria. The commonly used surfactants were Triton X-100, Tween 80, and Tween 20, to investigate its effect on extracellular lipase enzyme production. The result revealed that all surfactants could enhance lipase production significantly for approximately 2-fold, with the utilization of Tween-80 provided most promising result when it increased lipase activity to 0.221±0.001 U/mL/min during 24 hour, followed by Triton X-100, registered 0.189±0.003 U/mL/min and Tween-20, recording 0.179±0.028 U/mL/min at the corresponding within 24 hours of incubation period. According to (Immanuel et al. 2008), Tween-80 plays double role on biodegradation of engine oil.

It can induce the production of lipase enzyme besides playing role as an emulsifying agent which increases the solubility of engine oil in medium, rendering them more accessible for biodegradation. (Wu and Tsai 2004) also reported that higher level of lipase production could be achieved when Tween-80 was incorporated into a medium with lipidic substrate because it can emulsifying the oil, subsequently lower the interfacial tension between oils and water and resulting increase of cell permeability, thus possibly facilitates enzyme secretion. Referring to Figure 4.15, lipase activity was lower in the medium supplemented with Tween-20 and Triton X-100 during 48 and 72 hour of incubation as compared with control, which had no surfactant. This is might be due to the inhibition of surfactant on lipase production. (Koley and Bard 2010) reported that if cells are subject to prolonged exposure to Triton X-100, this chemical compound would disrupt hydrogen bonding present within the cell's lipid bilayer, leading to the destruction of the integrity of the lipid membrane.

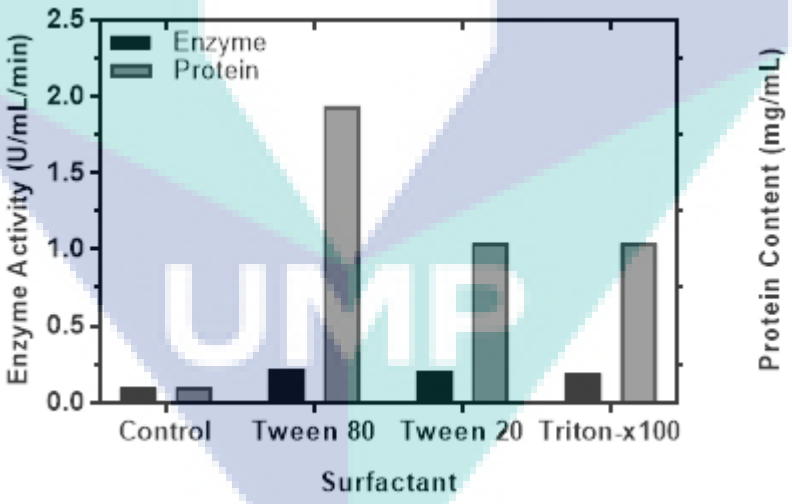

Figure 4.15 Effect of addition of different surfactants on lipase production and protein content by GS-3 isolate over 24 hours of incubation period

4.8.6 Effect of Medium Ingredients Removal on Lipase Production

Removal of $FeCl_3$ from production medium could enhance the lipase activity up to 0.142±0.048 U/mL/min after 24 hours of incubation while removal of $MgSO_4$ and $CaCl_2$ would adversely impact the lipase activity with protein content up to 0.572±0.013 mg/ml. figure (4.12) which prove that $MgSO_4$ and $CaCl_2$ are enzyme inducer .

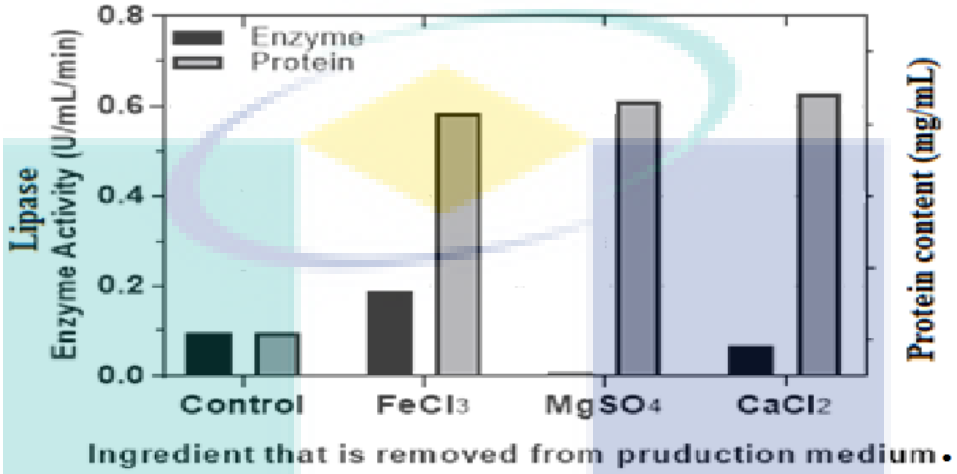

Figure 4.16 Effect of ingredient removal from production medium on lipase production and protein content over 24 hours of incubation period

The effect of trace element on lipase activity was studied. As previously discussed, lipase activity was generally low when disposed engine oil was used as sole carbon source. Besides, the exact chemical composition of disposed engine oil used as substrate for this study remains unknown. Therefore, it was worth to identify the compound in the production media that inhibit the lipase activity, especially trace element like Fe^{2+} and Ca^2. Figure 4.16 showed that removal of $FeCl_3$ from production medium could increase lipase activity. Approximately1.5-fold higher than control sample which produced lipase activity of 0.095±0.002 U/mL/min. Therefore, data revealed that $FeCl_3$ inhibited lipase enzyme of GS-3 isolate. Besides, $MgSO_4$ and $CaCl_2$ were essential nutrients for GS-3 isolate as removal of them from production medium significantly reduced the lipase activity. Without $CaCl_2$, lipase activity was compared with control sample throughout the incubation period. (Simons et al. 1999) reported that calcium ion was important for structural stabilization of lipase enzyme. Up to date, there is no consistent trend has been reported regarding the effect of metal ion on lipase activity (Shangguan et al., 2011). According to (Ali et al. 2009), $FeCl_3$ drastically inhibit lipase activity from *Aspergillus niger* at low concentration. Besides, Fe^{3+} ion also inhibited the lipase activity from *Bacillus subtilis* (Ma et al., 2006) and *Staphylococcus* sp at 200 ppm as per (Tembhurkar et al. 2012). However, some researches showed contradictory result.

For lipase enzyme, metal ion plays important role in structural property rather catalytic property. (Açikel et al. 2011) suggested that metal ion will inhibit lipase enzyme activity by reacting with –SH groups of lipase enzyme molecule through oxidation. (Liebeton et al. 2001) also remarked that presence of –SH groups in the lipase molecules resulting them to be intrinsically unstable. When metal ions react with –SH groups in the side groups of cysteine residues, tertiary structure of enzyme will be altered subsequently change .The shape of the active site and thus the enzyme becomes deactivated. Therefore, like nitrogen sources, the effect of trace elements on lipase activity varies among bacteria. (Appendix B).

4.9 Preliminary screening for biodegradation of DEO contaminated soil

The preliminary screening for biodegradation was carried out in order to determine the efficiency of toluene to extract the hydrocarbon before and after biodegradation. The control was used to determine the hydrocarbon present in the sample at zero time of incubation. While the three replicates were kept in incubator at 37°C for 4 days the concentration of hydrocarbon has been determined from 0.613 to0.041 and the percentage of the hydrocarbon degraded by the soil mixed culture were determined to be 93.3% after 4 days of incubation period. Figure (4.17).

Table 4.4

Total hydrocarbon content before and after biodegradation determined using Toluene as the best solvent.

Incubation Time	**Absorbance (Ab) at 286nm**	**Total Hydrocarbon Concentration (µl/ml)**	**Percentage of hydrocarbon degradation (%)**
Control	2.641	0.613	0
1	0.176	0.041	93.3
2	0.184	0.043	93.0
3	0.210	0.049	92.0

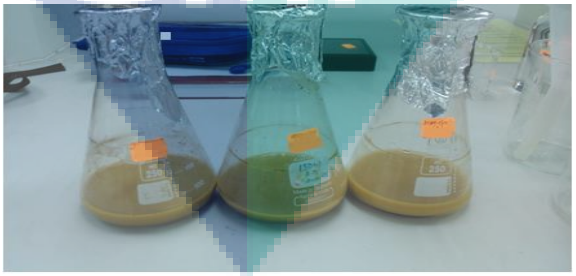

Figure 4.17 Three replicates of sample were prepared containing 1g of DEO contaminated soil in 50ml of MSM.

4.10 OFAT Method controlling biodegradation of DEO contaminated soil

One factor at time (OFAT) methods was used to choose the optimum parameters for bacterial degradation process and ethanol production. Using different conditions at the same time such as incubation period, initial pH-value, temperature, etc…

4.10.1 Effect of Incubation period

Data represented in Figure 4.18 demonstrated the effect of incubation period on the biodegradation of disposed engine oil. Based on the graph, as the incubation period increases the total hydrocarbon content in the sample began to decrease from the range of 0.013-0.004(µl/ml), while the biodegradation was increased from 0-69.23%. This is due to the presence of active microorganisms from the contaminated soil samples which will be doubled or tripled in numbers on the 10th day of incubation (Appendix B).

While (Wright, 1988) reported the maximum ethanol production of 4% (w/v) while converting the wheat straw to ethanol after 48 h of incubation employing process of simultaneous saccharifi cation and fermentation uses T. reesei cellulase and Kluyveromyces fragilis. tannophilus. Some authors have reported maximum ethanol yield after 48 h of incubation from starchy materials (Suresh et al., 1999 & Ratnam et al., 2003). The effect of incubation time and soil depth on the sorption of cesium on aerobic mineral soil.

The effect of time on the sorption of cesium is typically explained as the initial fast ion exchange on the easily accessible sorption sites followed by slow diffusion to the less available sites within the particles and interlayer sites of micas and clays, (Söderlund et al., 2016).

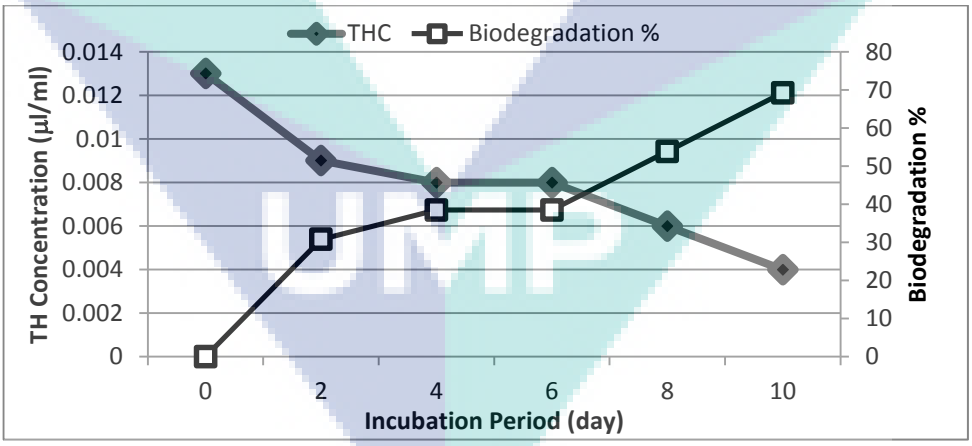

Figure 4.18 Shows the effect of incubation period on the hydrocarbon concentration and biodegradation %

4.10.2 Initial pH-value

Another parameter which plays vital role in microbial biodegradation of disposed engine oil is the pH-value of the degradation medium. Based on figure (4.19),

pH 7 showed the highest biodegradation percentage of the hydrocarbons compared to others up to 61.54%. This is due to the fact that soil microorganism grow well in neutral condition compared to acidic and alkaline environment. This is because such conditions reduce the metabolic activity of the soil microbial consortium. On the other hand, pH 6 seems to be the condition with lowest hydrocarbon degradation rate which was 15.38%.

Therefore the optimum pH required by soil microorganism was pH 7 for efficient growth and higher metabolic activity and degradation (Appendix B). (Kosaric et al., 1982), therefore, an initial pH of 5.5 was used in this work and the pH was not further controlled. Although the fermentations were run aseptically, the use of an acidic starting pH was intended to minimize the cost of an eventual commercial fermentation process: a low pH suppresses growth of unwanted microorganisms so that fermentation may be carried out without requiring the medium to be sterilized as sterilization is expensive in an industrial operation. These data meet those obtained by (Van Zyl et al., 1991), according to whom, using initial pH 6.50 may be an effective strategy to minimize acetic acid inhibitory effect and the lignin degradation compounds (phenols) of bioconversion by S. stipitis yeast. According to (Lu et al., 2017)pH did not significantly affect the metabolism of volatile fatty acids (VFAs) in all treatments with most of VFAs being metabolized to trace or undetectable levels after fermentation Aldehydes are intermediate compounds that can be metabolized by yeast cells during fermentation. pH did not significantly affect the metabolism of aldehydes . Aldehydes like acetaldehyde, hexanal, nonanal, benzaldehyde, and 4-tolualdehyde that were detected in the durian pulp were metabolized to trace or undetectable levels after fermentation.

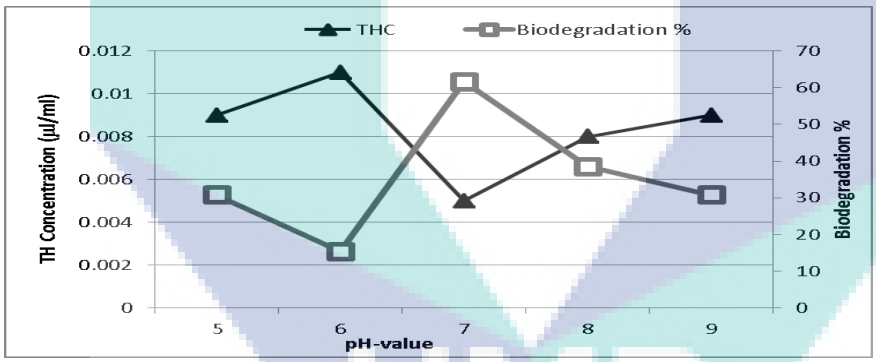

Figure 4.19 shows the effect of different pH-value on the biodegradation of hydrocarbon concentration and in DEO % sample.

4.10.3 Incubation Temperature

Apart from incubation period and pH, another crucial factor that affects the microbial biodegradation of used engine oil is the incubation temperature. The highest temperature used was 50°C and the lowest at 20°C. Different microorganism will show different adaptability toward the temperature. Some thermophilic bacteria enable to grow under high temperature. In this case, based on figure 4.20 the microbes able to perform well at 40°C where they able degrade the hydrocarbons in the soil by 69.23%. The second highest was at 50°C which indicates that there are some thermophilic bacteria that might present in the mixed culture of the soils. At 30°C, the microbial

biodegradation still occurs but in a slow state with 30.77% of hydrocarbon remediation (Appendix B). Verma et al., also reported 30°C as the optimum temperature for maximum ethanol production using starch employing co-culture of amylolytic yeast and S. cerevisiae. Thus, optimum temperature for simultaneous saccharifi cation and fermentation of kinnow waste and banana peels was found to be 30°C with maximum ethanol yield of 0.376 gg and fermentation efficiency of 74.11 % at 48 h of incubation. At temperatures lower or higher than optimum, less ethanol production was observed. Decline in ethanol yield at increased temperature might be due the inactivation of enzymes involved in ethanol production pathways. Increasing temperature also increases the maintenance energy demand (Fieschko and Humphrey, 1983). The known effects of ethanol on regulation of some of the genes in *Z.* mobilis (Yang et al., 2013) may also contribute to the observed changes in the biomass yield on glucose and the other kinetic parameters of the fermentation. According to (Lu et al., 2017) Temperature did not significantly affect the metabolism of volatile fatty acids (VFAs) in all treatments with most of VFAs being metabolized to trace or undetectable levels after fermentation. Aldehydes are intermediate compounds that can be metabolized by yeast cells during fermentation. Temperature and pH did not significantly affect the metabolism of aldehydes Aldehydes like acetaldehyde, hexanal, nonanal, benzaldehyde, and 4-tolualdehyde that were detected in the durian pulp were metabolized to trace or undetectable levels after fermentation. Lopez-Hidalgo et al.2017 has also reported that the effect of substrate concentration, temperature in production, production rate and yield of bioethanol and biohydrogen was evaluated with a Central Composite experimental design. Consequently, the experiment 18 showed the highest production of bioethanol and biohydrogen, with 8.0 g EtOH dm_3 and 509.2 cm3 H2, respectively. 0.054 g EtOH dm_3 h_1 and 25.27 cm3 H2 dm_3 h_1, were the highest production rates, and occurred when the working conditions were 15 g TRS dm_3, 37 _C Experiment 7 exhibited the highest yields, for bioethanol 0.51 g EtOH g TRS_1 and for biohydrogen 239.3 cm^3.

H2 g TRS_1. Biohydrogen production obtained by using WSH is higher than the one obtained with carbohydrates analytical grade; this phenomenon could be explained because WSH contains a mixture of carbohydrates and acid organics that E. coli can metabolize.

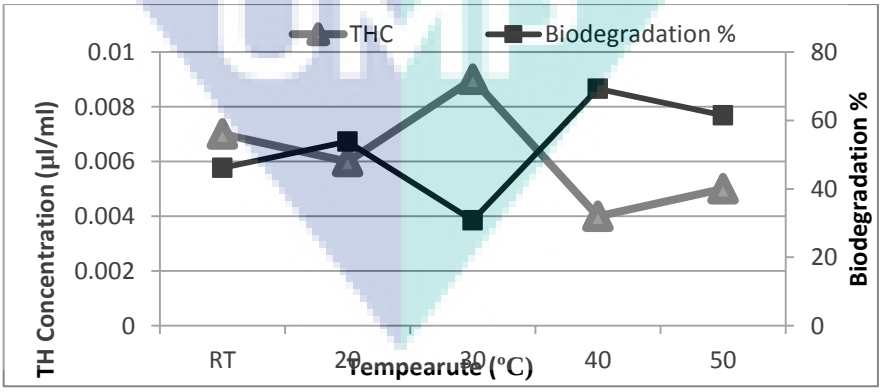

Figure 4.20 The graph represents the effect of temperature to words the biodegradation % of HC in DEO sample.

4.10.4 Effect of Inoculum Size

Inoculum size refers to the amount of soil added into the medium which indirectly indicates the amount of bacteria that present in the soil sample. The higher the amount of bacteria the higher the microbial biodegradation of the hydrocarbons. However,based on data represented in figure (4.21) it shows that the sample inoculated with 1g of soil shows the highest biodegradation percentange of 69.23% with concentration up to 0.004 (µl/ml) compared to the sample with 4g of soil which was the second highest with 67.31%. This might be contributed by the fact that 4g of soil might contain high amount of bacteria naturally, but there is also high amount of hydrocarbon in the contaminated soil sample. Thus it makes the bacteria to consume longer time to biodegrade the hydrocarbons. On the other hand, in 1g of soil the microbes able to degrade the small amount hydrocarbons present in the soil. Thus, the lesser the amount of hydrocarbon in the soil ,the higher the metabolic activity of the soil microorganism (Appendix B).Acoording to (Sharma et al., 2007) . The amount of sugar consumed and ethanol produced increased linearly with increase in initial cell concentration from 2.0 to 10% with 6% S. cerevisiae G (v/v) and 4% P. tannophilus MTCC 1077 (v/v) inoculum concentration producing maximum ethanol yield of 0.394 gg–1. The crude oil concentration plays an important role. For crude oil concentrations in water varying from 0.1 to 4.5 %, maximum degradation activity was found at a substrate concentration of 2 % (Sharma & Schiewer, 2016). Since Arctic shorelines have not yet been exposed to a major oil spill, little is known about crude oil biodegradation in that environment. Therefore, further research is necessary to evaluate under which conditions bioremediation of oilcontaminated arctic shoreline sediments can be effective, though sometimes results of laboratory studies cannot directly be scaled up, due to heterogeneity and concentration gradients in larger settings (Horel et al., 2015). The potential for crude oil bioremediation as part of an oil spill response along Arctic shorelines is evaluated in a microcosm experiment using sediments from Barrow, Alaska, investigating the combined effects of varying temperatures, crude oil concentrations, and salinities on crude oil biodegradation and fate. The research will assist decision-makers in choosing effective spill response strategies for future crude oil spills in Arctic shorelines.

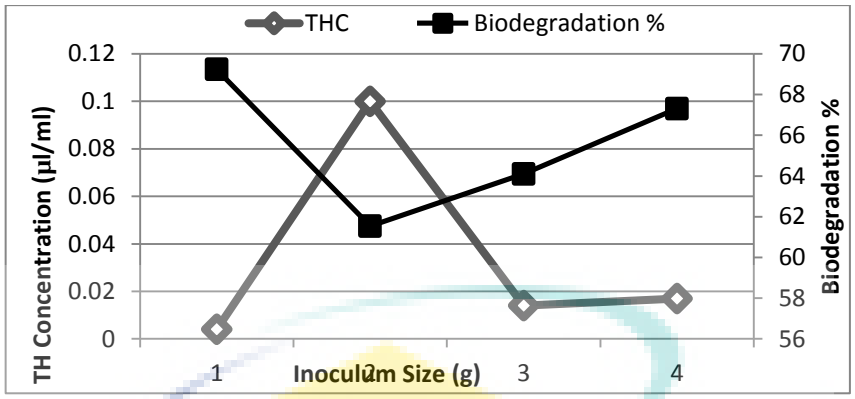

Figure 4.21 The graph shows the effect of different inoculum size on total hydrocarbon concentration in the sample.

4.10.5 Effect of Aeration

Aeration is another factor that can affect the biodegradation of DEO by incubating the samples under static or shaking conditions. This is usually related to the way of distributing the nutrients equally to the microorganism to grow well. However, based on Figure (4.22) it can be seen that static condition had shown highest biodegradation % rate up to 46.15% while shaker condition had given lower percentage 15.38% although shaking helps to enhance the mixture of nutrients and oxygen in the sample, too vigorous motion and continuously shaking motion might damage the microbial cells in the sample. Thus this will cause a tremendous reduction in the rate of biodegradation of hydrocarbon. At the same time, several studies mentioned that shaking was needed during the hydrocarbon extraction stage where it helps the hydrocarbon to mix well with the solvent used. Therefore according to the results obtained static condition was more favorable for the soil microorganism to perform well on bioremediation of DEO contaminated soil (Appendix B). (Sani & Dahman, 2010) Static conditions are better for keeping a regular shape and to maintain Using good morphology molds under static conditions, for example, can yield a product which is smooth and uniform, which is suitable for use (Fu et al., 2013).

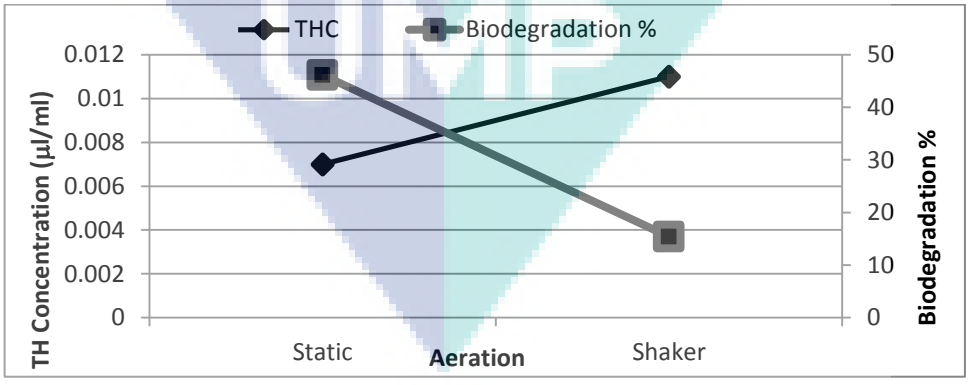

Figure 4.22 The graph shows the effect of aeration on the microbial degradation of hydrocarbons present in the oil contaminated soil sample.

4.10.6 Incubation condition

Oxygen is another important factor that enhances the microbial activity in biodegradation of DEO. Most soil microbial consortium requires oxygen for their growth and their metabolic activity if highly dependent on oxygen. Based on the result in Figure (4.23), aerobic condition was strongly preferred by the soil mixed culture in order to degrade the hydrocarbons with 76.92%. On the other hand, under anaerobic condition the rate of biodegradation of hydrocarbon was very low compared with the previous results as well. Thus, the presence of oxygen is the important requirement for the bioremediation of the engine oil contaminated soil samples (Appendix B). Oxygen enrichment of the atmosphere generally stimulates degradation of nonlignin components as well as lignin, so it increases the rate, but not the selectivity, of delignification (Reid and Seifert, 1980; Levonen-Munoz and Bone, 1985). Also O2 concentration can drop to rather low levels without completely abolishing lignin degradation.

Phlebia tremellosa degraded lignin in aspen wood well at O2 partial pressure as low as 7 kPa (Reid, 1985). The most important factor is a uniform distribution of air throughout the fermenting substrate and the ease of achieving this depends on the packing behavior of the substrate and the growth pattern of the fungus. Some fungi show thick mycelial growth binding the particles together, presumably block interparticle channel, and thereby increase stream resistance to air flow. In aerobic mineral soil, The retention of cesium increases with increasing time, for example from 3400 ml g-1 on day one to 6500 ml g-1 on day 245 for the sample depth of 0.7 m.

The average growth factor was 100 % (–13 to ?430 %) , corresponding to an increase in the Kd value of 2–5 fold. In anaerobic conditions , the average growth factor was approximately the same at 48 % (–37 to ?130 %). The effect of time on the sorption of cesium is typically explained as the initial fast ion exchange on the easily accessible sorption sites followed by slow diffusion to the less available sites within the particles and interlayer sites of micas and clays, (Söderlund et al.2016).

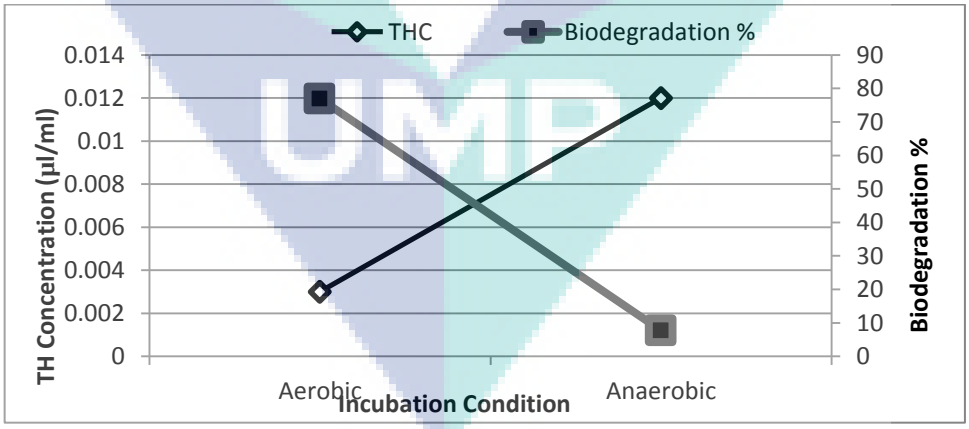

Figure 4.23 The graph demonstrates the effect of presence or absence of oxygen on biodegradation of disposed engine oil.

4.10.7 Effect of Flask Volume Capacities

The flask volume capacity is one of the new parameter added to observe its effect on the biodegradation of hydrocarbons found in the crude engine oil contaminated soil. Flask volume capacity involves the idea of the amount of trapped oxygen available for the microbial biodegradation. The fixed volume of 50ml of MSM with 1g of soil was added to different volume of flask from 50ml – 500ml. Based on the results in Figure 4.24 it shows that the highest oil degradation was take place in 500ml volume flask. This is due to the fact that have mention earlier that the wide surface area in 500ml volume flask for the 50ml of the sample will provide more trapped oxygen which would enhance the microbial degradation of the hydrocarbons. On the other hand, the lowest degradation rate occurred in the flask with small surface area with less trapped oxygen in it. Thus, 500ml flask was chosen to be used for further degradation steps (Appendix B).

Increasing the Flask volume capacity beyond the above mentioned concentration resulted as incline in fermentation yields which is in accordance with the results reported earlier (Kargi et al., 1985). The effect of O_2 on growth and glucose oxidase production in *A. niger* was studied by (Zetelaki and Vas, 1968). Increases in both these parameters were observed when increases in agitation rate were applied to a 10 liter fermenter culture, with further improvements achieved by using pure O_2, instead of air. The viscosity of the culture increased with the increased concentration of mycelium that resulted, and decreased with the appearance of autolysis, which also occurred more rapidly in these cultures Increases in sugar concentration in the more highly aerated cultures did not affect the growth and enzyme production rate, indicating that O_2, was the limiting, and therefore controlling, factor of the fermentation increasing the flask volume capacity can provide O_2 for the need of microorganism.

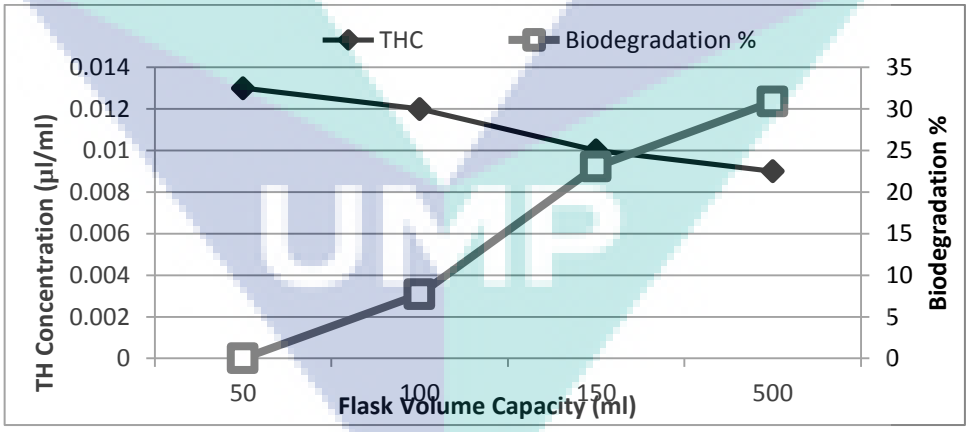

Figure 4.24 The graph shows the effect of flask volume capacity on the biodegradation of hydrocarbon present in the waste engine oil.

4.10.8 Effect of surfactant

Addition of surfactant is another factor that can contribute the efficient microbial biodegradation of DEO. This is due to the fact that oil does not dissolve in water.

Therefore surfactant plays an important role by adsorb at the interface of water and oil which enables the oil to be dissolved into the water. This will help the soil microbes to obtain the sufficient amount of carbon source from the hydrocarbon in the oil. Based on the results obtained in Figure 4.25 the highest percentage of biodegradation hydrocarbon were observed in the sample with Tween20 36.84%.With the same amount of surfactant added, Tween20 had shown the highest hydrocarbon degradation rate (Appendix B). Tween 20 has increased the biodegradation rate of waste engine oil in previous study done by (Maliji et al., 2013). However, majority of the papers have suggested that Tween80 to be the efficient surfactant that can be used for biodegradation of used engine oil as in (Das & Chandran, 2010; Montagnolli et al., 2015). According to Tian et al., (2016) The effect of one biosurfactant and four synthetic surfactants on crude oil biodegradation were tested. Experiments were carried out in 250 ml flasks containing 100 ml of mineral salts medium, supplemented with 0.5% (w/v) crude-oil. Those five surfactants were added to the flask cultures at five different concentrations (0.1 , 0.2 , 0.5, 1 and 2). 5% (v/v) seed culture as mentioned was inoculated into flask cultures. All the flasks were incubated at 30 °C, shaken at 160 rpm for 10 days. Un-inoculated flasks were served as control. After the biodegradation process, the crude oil extraction procedure was conducted as described by Varjani et al.,(2015). The solvent was allowed to evaporate and the total biodegradation rate was determined gravimetrically using the following formula: Biodegradation (%)¼[(WI-WC)/WI] 100, where WI is the weight of the residual oil in control and WC is the weight of the fraction in the culture. Each experiment was repeated three times and those results were expressed as the mean and standard deviation of three in dependent measurements. Kaczorek et al. (2015) also reported that the addition of surfactants became unnecessary for microorganisms with a high potential of hydrocarbon removal. During the cultivation process, crude oil in flasks without surfactants dispersed into tiny oil droplets, and surface tension of the culture medium reduced to 47.1 mn/m, indicating secretions of surface active agents were produced by microorganisms. From previous studies, surface active agents were usually biosurfactants, organic acids and biopolymers (Youssef et al., 2009). The time was shorter for crude oil emulsification in flasks supplemented with surfactants, usually within a few hours, thus the adaptation period of microorganisms could be greatly reduced. The residual oil extracted was analyzed quantitatively using GC–MS analysis. The total ion chromatogram (TIC) of oil samples. The crude oil was highly degraded and completely depleted of n-alkanes. Varjani et al. (2015) revealed lower alkanes of C8–C10 in crude oil were depleted 100% after 25 days incubation in control flasks, which indicated abiotic/physical factors might result in the removal of these components. crude oil had been strongly degraded, which could be concluded from the decreased peaks. The finding by Varjani et al. (2015) showed that 83.49% of crude oil biodegradation was conducted by HUBC (Hydrocarbon utilizing bacterial consortium) with gas chromatographic analysis.

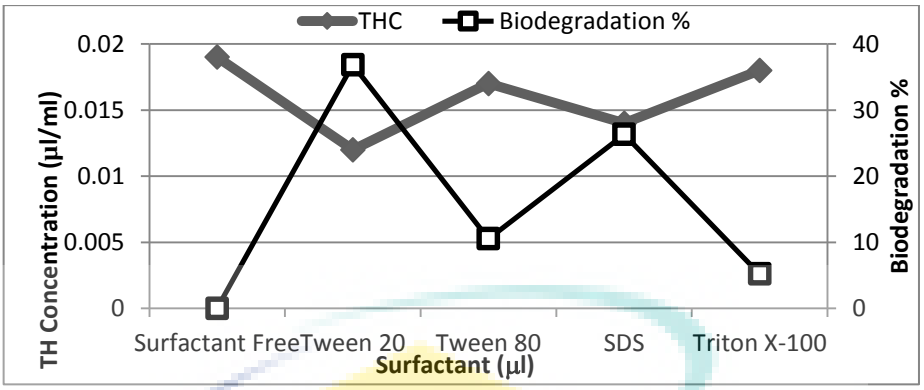

Figure 4.25 The graph shows the effect of surfactant on the biodegradation of disposed engine oil.

4.11. The Optimum Conditions for Biodegradation of DEO Contaminated Soil

Data summarized in table 4.5 showed that the optimum parameters has collected in a 500ml flask containing 50 ml MSM medium ,pH 7 ,1 gm of DEO contaminated soil ,Tween 20 and was kept under aerobic condition at 40ºC for 10 days. The biodegradation process was up to 69.24%.

Table 4.5 *The summary of optimum parameters and conditions required for efficient disposed engine oil degradation.*

No.	Parameter /50ml	Optimal Condition	Total Hydrocarbon Concentration (µl/ml)
1	Incubation period (days)	10	0.004
2	pH Initial-value	7	0.005
3	Temperature (°C)	40	0.004
4	Inoculum size (g)	1	0.004
5	Aeration	Static	0.007
6	Flask Volume Capacity (ml)	500	0.009
7	Incubation Condition	Aerobic	0.003
8	Surfactant (µl)	Tween 20	0.012
		Total/50ml	0.004

4.12 Gas Chromatography - Flame Ionization Detector (GC-FID) Analysis

The GC-FID results revealed that the aqueous layer extracted after biodegradation under optimum condition in 50ml and MSM for 10days and followed by another 10days of continuous fermentation in IL MSM were successfully produced ethanol. The percentage of ethanol in zero time of incubation was 2.27% and increased to 81.53% after ten days of incubation in 50ml of MSM medium. While the percentage

of ethanol continuously increased to 84.51% from the continuous fermentation of the 50ml MSM which was feed into 1L MSM. The results had given a positive outcome in which the main objective of the research was successfully achieved. Figure (4.26)(4.27)(4.28)(4.29).The ethanol is appeared at 1.634 minutes that compared to the ethanol standard which was at 1.636 minutes. However the extraction solvent peak was at 1.439 minutes. Regarding to Sharma & Schiewer (2016) the remaining crude oil in the sediments was determined using GC/FID. Triplicate 10 g sediment samples were taken from each jar. Crude oil was extracted from sediment samples using 25 ml methylene chloride. Twenty-five microliters of D- 5 nitrobenzene with a concentration of 2500 mg/l was used as an internal standard. Two hundred fifty microliters of D-8 naphthalene with a concentration of 2190 mg/l was added as a surrogate. The TPHs in the sediments were determined using a modified AK 102 and AK 103 methodology , using an Agilent Technologies 6890N Network GC coupled with flame ionization detector with column parameters 30 m by 250 µm by 0.25 µm and pulsed splitless injection with hydrogen as carrier gas (pressure 20 psi, flow 12.4 ml/ min, average velocity 15.2 cm/s). The oven temperature increased from 40 to 350 °C over 34.5 min. The total area of the chromatogram was taken into account when calculating the concentration of crude oil present in the sediments, using a standard calibration curve over the range of 500–5000 mg/l for highly concentrated samples and 175–700 mg/l for samples with lower crude oil concentration. The extraction efficiency of the surrogate D-8 naphthalene was calculated to be in the range of 80–95 %. To obtain the actual TPH values, the measured sample TPH concentration was divided by the crude oil recovery factor (measured initial TPH concentration/ theoretical initial crude oil concentration, which averaged 92 %).

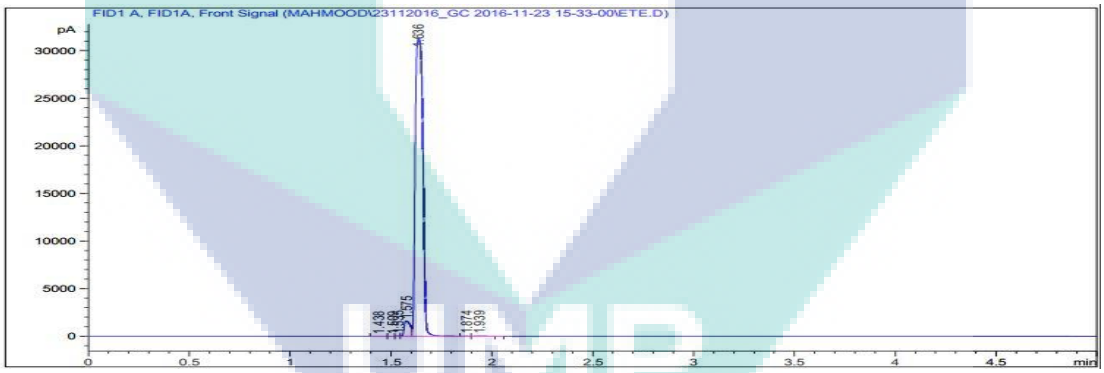

Figure 4.26 GC-FID spectrum obtained for the internal standard of ethanol solution.

Table 4.6 *The chemical components found in the internal standard used for GC-FID Analysis*

Peak	**Retention Time (min)**	**Compounds**	**Area (%)**
1	1.438	ND*	0.01
2	1.509	ND*	0.01
3	1.535	ND*	0.02

4	1.575	ND*	3.88
5	1.636	Ethanol	96.03
6	1.874	ND*	0.01

ND*: Not detected

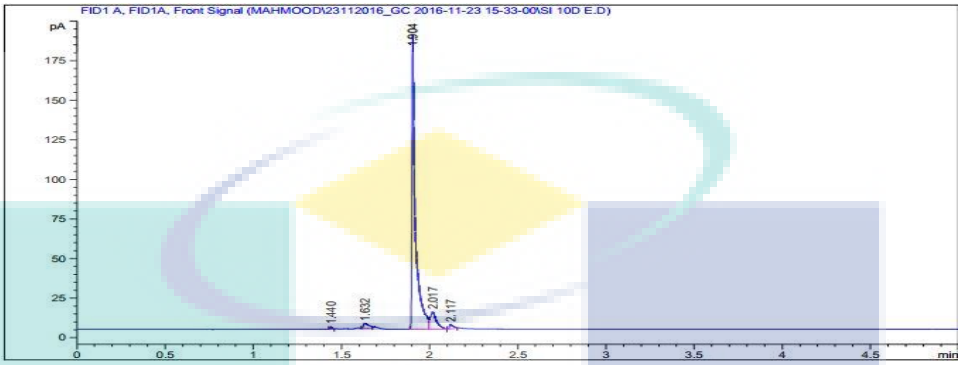

Figure 4.27 showed the DEO contaminated soil extracted sample at 0 time before incubation measured by GC-FID

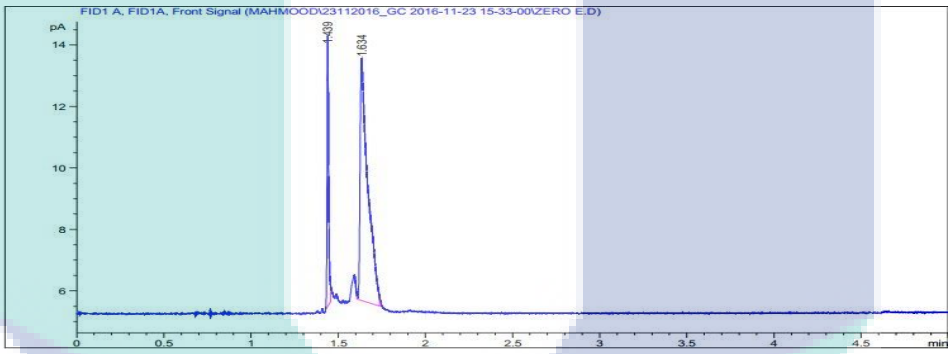

Figure 4.28 The GC-FID analysis for 50ml degradation medium after 10 days of incubation under optimum conditions.

Table 4.7 *The percentage of ethanol produced after 10days of incubation in 50ml degradation medium under optimum condition.*

Peak	**Retention Time (min)**	**Compounds**	**Area (%)**
1	1.439	Extraction solvent (toluene)	18.47
2	1.634	Ethanol	81.53

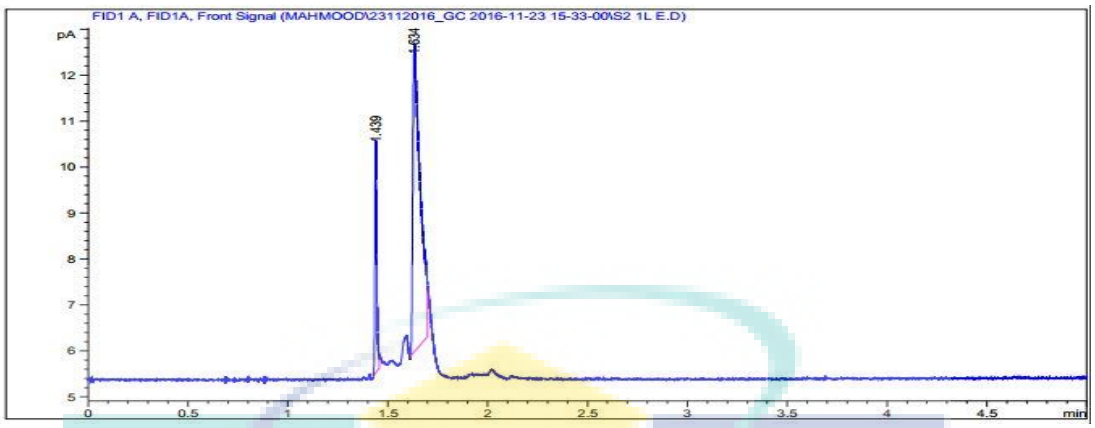

Figure 4.29 The GC-FID analysis obtained from one liter medium of DEO bioremediation through microbial fermentation.

Table 4.8 *The percentage of ethanol production from the continuous microbial fermentation of disposed engine oil in one liter MSM*

No	Retention Time (min)	Compound	Area (%)
1	1.439	Extraction solvent (toluene)	15.48
2	1.634	Ethanol	84.51

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Introduction

This chapter is to conclude the overall research outcome which had been successfully completed. Recommendations will be discussed in this chapter to improve future research.

5.2 Conclusion

This research project was successfully by carried out and all objectives were achieved. Hydrocarbon-degrading soil microorganisms such as bacteria and fungi were found in the oil-contaminated soil. It was able to produce alcohol such as ethanol through microbial fermentation of disposed engine oil. Ethanol has a huge application and demand as the biofuel and currently many industries looking for cheapest source for ethanol production. Thus this would be a great platform for the production of ethanol from disposed engine oil through microbial action. This research also demonstrated that optimization of certain experimental parameters are very important in enhancing the biodegradation of DEO. The highest rate of hydrocarbon degradation reached was 92.31% through continuous fermentation of DEO in 1L degradation medium. The optimum pH and temperature used for this experiment was pH 7 and 40°C respectively. Besides, addition of surfactant such as Tween 20 enhanced the biodegradation of the engine oil. Application of disposed water filter adds value to the degradation process by reducing the heavy metals and hydrocarbon present in the oil contaminated soil sample, meanwhile for Lipase production hydrocarbon-degrading bacteria, namely GS-3 was isolated from oil-contaminated soil. It was able to produce complex alcohol and organic acid from disposed engine oil. It is able secrete extracellular lipase, which is an industrially important by-enzyme. This research also demonstrated that optimization of production medium was very important in enhancing lipase activity. The best nitrogen source for maximum lipase production during first 24 hours was urea. Removal of $FeCl_3$ from production medium could increase lipase production to 0.142±0.048 U/mL/min, approximately1.5-fold higher than control, suggesting that $FeCl_3$ inhibit the lipase enzyme production. Addition of surfactant, tween 80 could enhance lipase production. Besides, optimal cultivation pH and temperature of isolate GS-3 for maximum lipase production was revealed to be pH 7 and 30°C respectively.

5.3 Recommendation

Although the soil mixed culture is able to degrade disposed engine oil and produce ethanol using disposed engine oil as sole carbon source, it need further study on the particular group of organism that contribute for ethanol production. This is because disposed engine oil contains hydrocarbon, which is not a favorable carbon sources. Thus, in order to increase the ethanol production we need to know the biodegrading agents that involved in this activity. Therefore, Although GS-3 is able to degrade disposed engine and produce extracellular lipase activity using disposed engine oil as sole carbon source, its lipase activity is considered low. This is because disposed engine oil contains hydrocarbon, which is not a favorable carbon sources. Further research should focus on increasing bioavailability of disposed engine oil to bacteria. For future research, it is also advisable to continue isolating novel bacteria or fungi which contributes for the ethanol and Lipase production from the disposed engine oil. Moreover the new approach of using disposed water filter in order to reduce the heavy metals found in the soil sample can be further studied in term of the mechanism involved and future applications involves it.

REFERENCES

Abioye, O. P., Agamuthu, P., & Abdul Aziz, A. R. (2012). Biodegradation of used motor oil in soil using organic waste amendments. Biotechnology research international, 2012.

Abioye, P. O., Aziz, A. A., & Agamuthu, P. (2010). Enhanced biodegradation of used engine oil in soil amended with organic wastes. *Water, Air, & Soil Pollution*, *209*(1-4), 173-179.

AÇIKEL, Ü., ERŞAN, M., & AÇIKEL, Y. S. (2011). The effects of the composition of growth medium and fermentation conditions on the production of lipase by R. delemar. *Turkish Journal of Biology*, *35*(1), 35-44.

Adewole, M. B., & Aboyeji, A. O. (2013). Yield and quality of maize from spent engine oil contaminated soils amended with compost under screenhouse conditions. *Journal of Agrobiology*, *30*(1), 9-19.

Alexander, M. (1994), Biodegradation and Bioremediation Academic Press Inc. *San Diego, California*, *267*, 269.

Ali, S., Huang, Z., Ren, S. X., Bashir, M. H., Afzal, M., & Tong, L. (2009). Production and extraction of extracellular lipase from entomopathogenic fungus Metarhizium anisopliae (Clavicipitaceae: Hypocreales). *Pak. J. Zool*, *41*, 341-347.

Atlas, R. M. (1992), Oil spills: regulation and biotechnology: guest editorial. *Current Opinion in Biotechnology*, *3*(3), 220-223.

Bagherzadeh-Namazi, A., Shojaosadati, S. A., & Hashemi-Najafabadi, S. (2009). Biodegradation of used engine oil using mixed and isolated cultures.

Ban, K., Kaieda, M., Matsumoto, T., Kondo, A., & Fukuda, H. (2001). Whole cell biocatalyst for biodiesel fuel production utilizing Rhizopus oryzae cells immobilized within biomass support particles. *Biochemical engineering journal*, *8*(1), 39-43.

Basuki, W., Syahputra, K., Suryani, A. T., & Pradipta, I. (2015). Biodegradation of Used Engine Oil by Acinetobacter junii TBC 1.2. *Indonesian Journal of Biotechnology*, *16*(2).

Bhangale, A. S., Beers, K. L., & Gross, R. A. (2012). Enzyme-catalyzed polymerization of end-functionalized polymers in a microreactor. *Macromolecules*, *45*(17), 7000-7008.

Burkert, J. F. D. M., Maugeri, F., & Rodrigues, M. I. (2004). Optimization of extracellular lipase production by Geotrichum sp. using factorial design. *Bioresource technology*, *91*(1), 77-84.

Butler, C. S., & Mason, J. R. (1996). Structure-function analysis of the bacterial aromatic ring-hydroxylating dioxygenases. *Advances in microbial physiology*, *38*, 47-84.

Chaturvedi, R., Prakash, J., & Awasthi, G. (2016). Microbial Bioremediation: An Advanced Approach for Waste Management.

Das, N., & Chandran, P. (2010). Microbial degradation of petroleum hydrocarbon contaminants: an overview. *Biotechnology research international*, *2011*.

Davis, M. W., Glaser, J. A., Evans, J. W., & Lamar, R. T. (1993). Field evaluation of the lignin-degrading fungus Phanerochaete sordida to treat creosote-contaminated soil. *Environmental science & technology*, *27*(12), 2572-2576.

Diaz, B. L., & Arm, J. P. (2003). Phospholipase A 2. *Prostaglandins, leukotrienes and essential fatty acids*, *69*(2), 87-97.

Dominguez-Rosado, E., & Pichtel, J. (2003, January). Chemical characterization of

fresh, used and weathered motor oil via GC/MS, NMR and FTIR techniques. In *Proceedings of the Indiana Academy of Science* (Vol. 112, No. 2, pp. 109-116).

Dongfeng, Z., Weilin, W., Yunbo, Z., Qiyou, L., Haibin, Y., & Chaocheng, Z. (2011). Study on isolation, identification of a petroleum hydrocarbon degrading bacterium Bacillus fusiformissp. and influence of environmental factors on degradation efficiency. Environment Protection, 13(4), 74-82.

Egmond, M. R., & van Bemmel, C. J. (1997). [6] Impact of structural information on understanding lipolytic function. *Methods in enzymology*, *284*, 119-129.

Ejoh, O. E., Adenipekun, C. O., & Ogunjobi, A. A. (2012). Effect of Pleurotus tuber-regium Singer and microorganisms on degradation of soil contaminated with spent cutting fluid. *New York Sci. J*, *5*(10), 121-128.

Fu, L., Zhang, J., & Yang, G. (2013). Present status and applications of bacterial cellulose-based materials for skin tissue repair. *Carbohydrate polymers*, *92*(2), 1432-1442.

Fu, L., Zhou, P., Zhang, S., & Yang, G. (2013). Evaluation of bacterial nanocellulose-based uniform wound dressing for large area skin transplantation. *Materials Science and Engineering: C*, *33*(5), 2995-3000.

Gadd, G. M. (2010). Metals, minerals and microbes: geomicrobiology and bioremediation. *Microbiology*, *156*(3), 609-643.

Garlapati, V. K., Shankar, U., & Budhiraja, A. (2016). Bioconversion technologies of crude glycerol to value added industrial products. Biotechnology Reports, 9, 9-14.

Girod, A., Wobus, C. E., Zádori, Z., Ried, M., Leike, K., Tijssen, P., .. & Hallek, M. (2002). The VP1 capsid protein of adeno-associated virus type 2 is carrying a phospholipase A2 domain required for virus infectivity. *Journal of General Virology*, *83*(5), 973-978.

Goñi, F. M., & Alonso, A. (2002). Sphingomyelinases: enzymology and membrane activity. *FEBS letters*, *531*(1), 38-46.

Guillén, M. D., & Cabo, N. (2000). Some of the most significant changes in the Fourier transform infrared spectra of edible oils under oxidative conditions. *Journal of the Science of Food and Agriculture*, *80*(14), 2028-2036

Guo, Z., & Xu, X. (2005), New opportunity for enzymatic modification of fats and oils with industrial potentials. *Organic & biomolecular chemistry*, *3*(14), 2615-2619.

Gupta, R., Gupta, N., & Rathi, P. (2004), Bacterial lipases: an overview of production, purification and biochemical properties. *Applied microbiology and biotechnology*, *64*(6), 763-781.

Gupta, S., Pathak, B., & Fulekar, M. H. (2015). Molecular approaches for biodegradation of polycyclic aromatic hydrocarbon compounds: a review. Reviews in Environmental Science and Bio/Technology, 14(2), 241-269.

Harding, K. G., Dennis, J. S., Von Blottnitz, H., & Harrison, S. T. L. (2008). A life-cycle comparison between inorganic and biological catalysis for the production of biodiesel. *Journal of cleaner production*, *16*(13), 1368-1378.

Horel A, Schiewer S, Misra D (2015). Effect of concentration gradients on biodegradation in bench scale sand columns with HYDRUS modeling of hydrocarbon transport and degradation. Environ Sci Pollut Res 22:13251–13263. doi:10.1007/s11356-015-4576-6

Huang, H., Bowler, B. F., Zhang, Z., Oldenburg, T. B., & Larter, S. R. (2003). Influence of biodegradation on carbazole and benzocarbazole distributions in oil columns from the Liaohe basin, NE China. *Organic Geochemistry*, *34*(7), 951-969.

Hurst, C. J., Crawford, R. L., Garland, J. L., & Lipson, D. A. (Eds.). (2007). *Manual of environmental microbiology*. American Society for Microbiology Press.

Ibrahim, H. M. (2016). Biodegradation of used engine oil by novel strains of Ochrobactrum anthropi HM-1 and Citrobacter freundii HM-2 isolated from oil-contaminated soil. *3 Biotech*, *6*(2), 226.

Immanuel, G., Esakkiraj, P., Jebadhas, A., Iyapparaj, P., & Palavesam, A. (2008). Investigation of lipase production by milk isolate Serratia rubidaea. *Food Technology and Biotechnology*, *46*(1), 60.

Jesubunmi, C. O. (2014). Isolation of oil-degrading microorganisms in spent engine oil-contaminated soil. J. Biol. Agric. Healthcare, 4(25), 191-195.

Jones, D. M., Head, I. M., Gray, N. D., Adams, J. J., Rowan, A. K., Aitken, C. M. & Oldenburg, T. (2008). Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. *Nature*, *451*(7175), 176-180.

Kaczorek, E., Smułek, W., Zgoła-Grześkowiak, A., Bielicka-Daszkiewicz, K., & Olszanowski, A. (2015). Effect of Glucopon 215 on cell surface properties of Pseudomonas stutzeri and diesel oil biodegradation. *International Biodeterioration & Biodegradation*, *104*, 129-135.

Kargi, F., Curme, J. A., & Sheehan, J. J. (1985). Solid-state fermentation of sweet sorghum to ethanol. *Biotechnology and bioengineering*, *27*(1), 34-40.

Koley, D., & Bard, A. J. (2010). Triton X-100 concentration effects on membrane permeability of a single HeLa cell by scanning electrochemical microscopy (SECM). *Proceedings of the National Academy of Sciences*, *107*(39), 16783-16787.

Kumar, A., Bisht, B. S., Joshi, V. D., & Dhewa, T. (2011). Review on Bioremediation of Polluted Environment:: A Management Tool. *International journal of environmental sciences*, *1*(6), 1079.

Kundu, S., Bhangale, A. S., Wallace, W. E., Flynn, K. M., Guttman, C. M., Gross, R. A., & Beers, K. L. (2011). Continuous flow enzyme-catalyzed polymerization in a microreactor. *Journal of the American Chemical Society*, *133*(15), 6006-6011.

Kupareva, A., Mäki-Arvela, P., Grénman, H., Eränen, K., Sjöholm, R., Reunanen, M., & Murzin, D. Y. (2012). Chemical characterization of lube oils. Energy & Fuels, 27(1), 27-34.

Kuppusamy, S., Thavamani, P., Venkateswarlu, K., Lee, Y. B., Naidu, R., & Megharaj, M. (2017). Remediation approaches for polycyclic aromatic hydrocarbons (PAHs) contaminated soils: Technological constraints, emerging trends and future directions. *Chemosphere*, *168*, 944-968.

Lee, L. P., Karbul, H. M., Citartan, M., Gopinath, S. C., Lakshmipriya, T., & Tang, T. H. (2015). Lipase-secreting Bacillus species in an oil-contaminated habitat: promising strains to alleviate oil pollution. *BioMed research international*, *2015*.

Liao, Y., Shi, Q., Hsu, C. S., Pan, Y., & Zhang, Y. (2012). Distribution of acids and nitrogen-containing compounds in biodegraded oils of the Liaohe Basin by negative ion ESI FT-ICR MS. *Organic geochemistry*, *47*, 51-65.

Liebeton, K., Zacharias, A., & Jaeger, K. E. (2001). Disulfide Bond in Pseudomonas aeruginosaLipase Stabilizes the Structure but Is Not Required for Interaction with Its Foldase. *Journal of bacteriology*, *183*(2), 597-603.

Lopez-Hidalgo, A. M., Sánchez, A., & De León-Rodríguez, A. (2017). Simultaneous production of bioethanol and biohydrogen by Escherichia coli WDHL using wheat straw hydrolysate as substrate. *Fuel*, *188*, 19-27.

Lowe, M. E. (1992). The catalytic site residues and interfacial binding of human pancreatic lipase. *Journal of Biological Chemistry*, *267*(24), 17069-17073.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of biological chemistry*, *193*(1), 265-275.

Lu, Y., Voon, M. K. W., Huang, D., Lee, P. R., & Liu, S. Q. (2017). Combined effects of fermentation temperature and pH on kinetic changes of chemical constituents of durian wine fermented with Saccharomyces cerevisiae. *Applied microbiology and biotechnology*, *101*(7), 3005-3014.

Mahmoud, G. A. E., Koutb, M. M., Morsy, F. M., & Bagy, M. M. (2015). Characterization of lipase enzyme produced by hydrocarbons utilizing fungus Aspergillus terreus. *European Journal of Biological Research*, *5*(3), 70-77.

Makkar, R. S., Cameotra, S. S., & Banat, I. M. (2011). Advances in utilization of renewable substrates for biosurfactant production. *AMB express*, *1*(1), 5.

Maliji, D., Olama, Z., & Holail, H. (2013). Environmental studies on the microbial degradation of oil hydrocarbons and its application in Lebanese oil polluted coastal and marine ecosystem. *Int J Curr Microbiol App Sci*, *2*(6), 1-18.

Mazumdar, S., Clomburg, J. M., & Gonzalez, R. (2010). Escherichia coli strains engineered for homofermentative production of D-lactic acid from glycerol. *Applied and environmental microbiology*, *76*(13), 4327-4336.

Meredith, W., Kelland, S. J., & Jones, D. M. (2000). Influence of biodegradation on crude oil acidity and carboxylic acid composition. *Organic Geochemistry*, *31*(11), 1059-1073.

Montagnolli, R. N., Lopes, P. R. M., & Bidoia, E. D. (2015), Assessing Bacillus subtilis biosurfactant effects on the biodegradation of petroleum products., *Environmental monitoring and assessment*, *187*(1), 4116.

Murarka, A., Dharmadi, Y., Yazdani, S. S., & Gonzalez, R. (2008). Fermentative utilization of glycerol by Escherichia coli and its implications for the production of fuels and chemicals. *Applied and environmental microbiology*, *74*(4), 1124-1135.

Narasimha, G., Kumar, A. P., & Subramanyam, D. (2011). Production and optimization of lipase enzyme by Pseudomonas sps. *BioTechnology: An Indian Journal*, *5*(1).

Obayori, O. S., Salam, L. B., & Ogunwumi, O. S. (2014). Biodegradation of fresh and used engine oils by Pseudomonas aeruginosa LP5. *Journal of Bioremediation & Biodegredation*, *5*(1), 1.

Obini, U., Okafor, C. O., & Afiukwa, J. N. (2013). Determination of levels of polycyclic aromatic hydrocarbons in soil contaminated with spent motor Engine oil in Abakaliki Auto-Mechanic Village. *Journal of Applied Sciences and Environmental Management*, *17*(2), 169-175.

Ogunbayo, A. O., Bello, R. A., & Nwagbara, U. (2012). Bioremediation of engine oil contaminated site. *Journal of Emerging Trends in Engineering and Applied Sciences (JETEAS)*, *3*(3), 483-489.

Oh, B. R., Seo, J. W., Heo, S. Y., Hong, W. K., Luo, L. H., Joe, M. H., ... & Kim, C. H. (2011). Efficient production of ethanol from crude glycerol by a Klebsiella pneumoniae mutant strain. *Bioresource technology*, 102(4), 3918-3922.

Onuoha, S. C., Olugbue, V. U., Uraku, J. A., & Uchendu, D. O. (2011). Biodegradation potentials of hydrocarbon degraders from waste lubricating oil-spilled soils in

Ebonyi State, Nigeria. Int. J. Agric. Biol, 13, 586-590.
Pereira, J. F., Gudiña, E. J., Costa, R., Vitorino, R., Teixeira, J. A., Coutinho, J. A., & Rodrigues, L. R. (2013). Optimization and characterization of biosurfactant production by Bacillus subtilis isolates towards microbial enhanced oil recovery applications. *Fuel*, *111*, 259-268.

Rao, M. A., Scelza, R., Scotti, R., & Gianfreda, L. (2010). Role of enzymes in the remediation of polluted environments. *Journal of soil science and plant nutrition*, *10*(3), 333-353.
Ratnam, B. V. V., Rao, M. N., Rao, M. D., Rao, S. S., & Ayyanna, C. (2003). Optimization of fermentation conditions for the production of ethanol from sago starch using response surface methodology. *World Journal of Microbiology and Biotechnology*, *19*(5), 523-526.
Rejitha, R., Devika, S., Balakumaran, M. D., & Nancy, A. I. (2012). Production, optimization and purification of lipase from Bacillus sp. MPTK 912 isolated from oil mill effluent.
Rigas, F., Papadopoulou, K., Philippoussis, A., Papadopoulou, M., & Chatzipavlidis, J. (2009). Bioremediation of lindane contaminated soil by Pleurotus ostreatus in non sterile conditions using multilevel factorial design. *Water, air, and soil pollution*, *197*(1-4), 121-129.
Ross, A. S., Farrimond, P., Erdmann, M., & Larter, S. R. (2010). Geochemical compositional gradients in a mixed oil reservoir indicative of ongoing biodegradation. *Organic Geochemistry*, *41*(3), 307-320.
Sadouk, Z., Tazerouti, A., & Hacene, H. (2009). Biodegradation of diesel oil and production of fatty acid esters by a newly isolated Pseudomonas citronellolis KHA. *World Journal of Microbiology and Biotechnology*, *25*(1), 65-70.
Sagar, K., Bashir, Y., Phukan, M. M., & Konwar, B. K. (2013). Isolation of lipolytic bacteria from waste contaminated soil: A study with regard to process optimization for lipase. Int. J. Scient. Technol. Res, 2, 214-218.
Sani, A., & Dahman, Y. (2010). Improvements in the production of bacterial synthesized biocellulose nanofibres using different culture methods. *Journal of Chemical Technology and Biotechnology*, *85*(2), 151-164.
Shangguan, J. J., Liu, Y. Q., Wang, F. J., Zhao, J., Fan, L. Q., Li, S. X., & Xu, J. H. (2011). Expression and characterization of a novel lipase from Aspergillus fumigatus with high specific activity. *Applied Biochemistry and Biotechnology*, *165*(3-4), 949-962.
Sharma, A., Kumar, P., & Rehman, M. B. (2014). Biodegradation of diesel hydrocarbon in soil by bioaugmentation of Pseudomonas aeruginosa: A laboratory scale study. International Journal of Environmental Bioremediation & Biodegradation, 2(4), 202-212.
Sharma, N., Kalra, K. L., Oberoi, H. S., & Bansal, S. (2007). Optimization of fermentation parameters for production of ethanol from kinnow waste and banana peels by simultaneous saccharification and fermentation. *Indian Journal of Microbiology*, *47*(4), 310-316.
Sharma, P., & Schiewer, S. (2016). Assessment of crude oil biodegradation in arctic seashore sediments: effects of temperature, salinity, and crude oil concentration. *Environmental Science and Pollution Research*, *23*(15), 14881-14888

Shukla, K. P., Singh, N. K., & Sharma, S. (2010). Bioremediation: developments, current practices and perspectives. *Genet. Eng. Biotechnol. J*, *3*(8), 1-20.

Söderlund, M., Virtanen, S., Välimaa, I., Lempinen, J., Hakanen, M., & Lehto, J. (2016). Sorption of cesium on boreal forest soil II. The effect of time, incubation conditions, pH and competing cations. *Journal of Radioanalytical and Nuclear Chemistry*, *309*(2), 647-657

Soetan, K. O. (2011). The role of biotechnology towards attainment of a sustainable and safe global agriculture and environment–A review. *Biotechnology and Molecular Biology Review*, *6*(5), 109-117.

Suhaimi, S. N., Phang, L. Y., Maeda, T., Abd-Aziz, S., Wakisaka, M., Shirai, Y., & Hassan, M. A. (2012). Bioconversion of glycerol for bioethanol production using isolated Escherichia coli SS1. Brazilian Journal of Microbiology, 43(2), 506-516.

Sujatha, K., & Dhandayuthapani, K. (2013). optimization of lipase production media parameters by a newly isolated bacillus licheniforms kdp from oil mill soil. *Int. J. Pharm. Bio. Sci*, *4*(2), 645-652.

Suresh, K., & Rao, L. V. (1999). Utilization of damaged sorghum and rice grains for ethanol production by simultaneous saccharification and fermentation. *Bioresource Technology*, *68*(3), 301-304.

Tembhurkar, V., Dama, L., Attarde, N., & Zope, P. (2012). Production and characterization of extracellular lipases of staphylococcus sp. isolated from oil contaminated soil. *Trends in Biotechnology Research*, *1*, 36-41.

Thenmozhi, R., Arumugam, K., Nagasathya, A., Thajuddin, N., & Paneerselvam, A. (2013). Studies on Mycoremediation of used engine oil contaminated soil samples. *Adv. in Applied Sc. Res*, *4*(2), 110-118.

Tian, W., Yao, J., Liu, R., Zhu, M., Wang, F., Wu, X., & Liu, H. (2016). Effect of natural and synthetic surfactants on crude oil biodegradation by indigenous strains. *Ecotoxicology and environmental safety*, *129*, 171-179.

Van de Voort, F. R. (1992). Fourier transform infrared spectroscopy applied to food analysis. *Food Research International*, *25*(5), 397-403

Varjani, S. J., Rana, D. P., Jain, A. K., Bateja, S., & Upasani, V. N. (2015). Synergistic ex-situ biodegradation of crude oil by halotolerant bacterial consortium of indigenous strains isolated from on shore sites of Gujarat, India. *International Biodeterioration & Biodegradation*, *103*, 116-124.

Venosa, A. D., & Zhu, X. (2003). Biodegradation of crude oil contaminating marine shorelines and freshwater wetlands. Spill Science & Technology Bulletin, 8(2), 163-178.

Vidali, M. (2001). Bioremediation. an overview. *Pure and Applied Chemistry*, *73*(7), 1163-1172.

Wang, W. D., Huang, Y. M., Shu, W. Q., & Cao, J. (2007). Multiwalled carbon nanotubes as adsorbents of solid-phase extraction for determination of polycyclic aromatic hydrocarbons in environmental waters coupled with high-performance liquid chromatography. *Journal of Chromatography A*, *1173*(1), 27-36.

Wu, H. S., & Tsai, M. J. (2004). Kinetics of tributyrin hydrolysis by lipase. *Enzyme and microbial technology*, *35*(6), 488-493.

Yang, H., Irudayaraj, J., & Paradkar, M. M. (2005). Discriminant analysis of edible oils and fats by FTIR, FT-NIR and FT-Raman spectroscopy. *Food Chemistry*, *93*(1), 25-32

Yang, S., Pan, C., Tschaplinski, T. J., Hurst, G. B., Engle, N. L., Zhou, W., ... & Johnson, C. M. (2013). Systems biology analysis of Zymomonas mobilis ZM4 ethanol stress responses. *PLoS One*, *8*(7), e68886.

Yazdani, S. S., & Gonzalez, R. (2008). Engineering Escherichia coli for the efficient conversion of glycerol to ethanol and co-products. *Metabolic engineering*, 10(6), 340-351.

Yong, R. N. (2000). *Geoenvironmental engineering:* Contaminated soils, pollutant fate, and mitigation. CRC press.

Yong, R. N., Mohamed, A. M. O., & Warkentin, B. P. (1992), Principles of contaminant transport in soils. *Elsevier Science Publishers*.

Youssef, N., Elshahed, M. S., & McInerney, M. J. (2009). Microbial processes in oil fields: culprits, problems, and opportunities. *Advances in applied microbiology*, *66*, 141-251.

Zakaria, M. P., Takada, H., Tsutsumi, S., Ohno, K., Yamada, J., Kouno, E., & Kumata, H. (2002). Distribution of polycyclic aromatic hydrocarbons (PAHs) in rivers and estuary

Zetelaki, K., & Vas, K. (1968). The role of aeration and agitation in the production of glucose oxidase in submerged culture. *Biotechnology and bioengineering*, *10*(1), 45-59

LIST OF PUPLICATIONS

1. **Mahmood H. Mahmood**, Zhi yang, Raid D. Thanoon, Essam A. Makky, Mohd Hasbi Ab. Rahim. (2017**) ,**Lipase production and optimization from ioremediation of disposed engine oil Journal of Chemical and Pharmaceutical Research, 9(6):26-36

2. **Mahmood H. Mahmood**, Rishi Ravee, Raid D. Thanoon, Essam A. Makky, Mohd Hasbi Ab. Rahim. (2017**) ,** biodegradation of disposed engine oil for ethanol production. *Journal of biotechnology*. **(Under Review)**

4. **Mahmood H. Mahmood**, Zhi yang, Raid D. Thanoon, Essam A. Makky, Mohd Hasbi Ab. Rahim. Bioremediation Of Disposed Engine Oil For Lipase Production. FGIC 1st Conference on Governance & Integrity, 2017 "Innovation & Sustainability Through Governance" 3 – 4 April 2017, Yayasan Pahang, Kuantan, Malaysia ISBN 978-967-2054-37-5

AWARDS

Mahmood H. Mahmood, Biodegradation of dispose engine oil for ethanol production . *Citrix 2017 UMP GAMBANG(Bronze Medal).*