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ชื่อโครงการ Hydrocarbon Degradation Potentials of Some Bacteria Species Isolated from Contaminated Soil with Spent Lubricating Oil

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Senior Project

Project Title	Hydrocarbon Degradation Potentials of Some Bacteria Species Isolated from Contaminated Soil with Spent Lubricating Oil
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Department	Environmental Science
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ประสิทธิภาพในการย่อยสลายสารประกอบไฮโดรคาร์บอนของแบคทีเรียบางชนิดที่แยกได้จากดินที่ปนเปื้อนน้ำมันหล่อลื่นที่ใช้แล้ว

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Hydrocarbon Degradation Potentials of Some Bacteria Species Isolated from Contaminated Soil with Spent Lubricating Oil

Pannathat Tansawat

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Accepted by the Department of Environmental Science, Faculty of Science, Chulalongkorn University in Partial Fulfilment of the Requirements for the Bachelor's degree

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หัวข้อ	ประสิทธิภาพในการย่อยสลายสารประกอบไฮโดรคาร์บอน โดยแบคทีเรียบางชนิดที่แยกได้จากดินที่ปนเปื้อนน้ำมันหล่อลื่นที่ใช้แล้ว
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บทคัดย่อ

งานวิจัยนี้มุ่งเน้นที่จะคัดเลือกแบคทีเรียที่มีความสามารถในการย่อยสลายน้ำมันหล่อลื่นที่ใช้แล้ว พร้อมทั้งตรวจวัดแก๊สคาร์บอนไดออกไซด์ที่เกิดจากกิจกรรมของแบคทีเรียในการย่อยสลายน้ำมันหล่อลื่นและศึกษาลักษณะสัณฐานวิทยาของแบคทีเรียที่แยกได้จากดินที่มีการปนเปื้อนน้ำมันหล่อลื่น ทั้งนี้ได้ทำการเก็บตัวอย่างดินมาจากอู่ซ้อมรถยนต์จังหวัดเชียงใหม่ พบว่าสามารถคัดแยกเชื้อแบคทีเรียได้ 4 สายพันธุ์ ทำการคัดแยกเบื้องต้นจากการนำดินตัวอย่างมา 10 กรัม ทำการละลายในน้ำกลั่น 90 มิลลิลิตร จากนั้นนำ 10 มิลลิลิตร ใส่ลงในอาหารเลี้ยงเชื้อ BHM 100 มิลลิลิตร (BHM + lubricating oil 1% + Tween 20 1%) นำไปบ่มเขย่าที่ 100 รอบต่อนาที ที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 24 – 72 ชั่วโมง ทำซ้ำทั้งหมด 3 ครั้ง จากนั้นนำเชื้อที่ได้มาเจือจางตั้งแต่ 10^{-1}-10^{-7} แล้วทำการเกลี่ยเชื้อให้ทั่วบริเวณผิวของอาหารเลี้ยงเชื้อ Trypticase Soy Agar หรือ TSA โดยอาศัยเทคนิค Spread Plate Technique นำไปบ่มที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 24 – 48 ชั่วโมง หลังจากนั้นทำการคัดเลือกเชื้อแบคทีเรียด้วยการทำให้เชื้อบริสุทธิ์ โดยอาศัยเทคนิค Streaking Plate Technique นำไปบ่มที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง ต่อมาได้ทำการย้อมแกรมของแบคทีเรียที่แยกได้พบว่าแบคทีเรียทั้ง 4 สายพันธุ์ เป็นแบคทีเรียแกรมลบ มีรูปร่างเป็นวงกลม นูนโค้ง ขอบเรียบ สีขาวขุ่น มีลักษณะเหมือนกันทั้ง 4 สายพันธุ์

คำสำคัญ: ไฮโดรคาร์บอน; น้ำมันหล่อลื่น; การปนเปื้อนในดิน; การย่อยสลาย; การเสื่อมสลายทางชีวภาพ

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ABSTRACT

This research aims to select bacteria that can biodegrading of used lubricating, measures carbon dioxide from bacterial activity in the degradation of lubricants, and study morphology of bacteria that isolated from contaminated soil with lubricants and collect the example of soil from a garage in Chiang Mai. This research found that could be isolated 4 strains of bacteria by take 10 g of sample soil and dissolve in distilled water 90 ml. Then, take 10 ml in culture medium BHM 100 ml (BHM + lubricating oil 1% + Tween 20 1%) and shake 100 rpm at 37 degrees for 24-72 hr. repeat 3 times.

After that, take the bacteria dilute from 10^{-1}-10^{-7}, and spread it on the surface of Trypticase Soy Agar or TSA by Spread Plate Technique. Then, incubate at 37 degrees for 24-48 hr. After that, selecting bacteria for purification by Streaking Plate Technique and incubate at 37 degrees for 24 hr. Then, gram staining all 4 bacteria. This research found that all of them are gram negative bacteria, entire (smooth), convex, curve, and opaque.

Keywords: hydrocarbon; lubricating oil; contaminated soil; degradation; bioremediation

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CHAPTER 1

Introduction

1.1 Background & Rationale

Lubricating oil is Hydrocarbon compounds that normally ranges from about carbon C_{20} to C_{35} but can be as low as C_{15} and as high as C_{50} by Fractional distillation. Crude Oil is separated into fractions by heating process known as distillation. The refinery cleans and separates crude oil into fuel and a by-product of it is lubricant. This process is based on parts of the crude oil that are difference of both boiling point and condensation point. Crude oil will be pumped into the furnace which is the temperature is high enough for every part of it to vaporization. Then, the vapor is sent to fractionating tower cylinder. Inside the fractionating tower cylinder are divided into different into chambers by plates, each of which has perforated to allow the crude oil vapor pass through the upper part of the fractionating tower. Moreover, there is an extension tube that takes the refined oil out of the fractionating tower. While the oil vapor is moving, the temperature of it decreases and condense. Each part of the oil vapor becomes liquid differently levels. It depends on the boiling point and condensation temperature that is different according to the number of carbon atoms in the molecule. Compounds with a small amount of carbon evaporate and condense to liquid in the upper layers of the fractionating tower. However, compounds with a large number of carbon atoms in molecules and high boiling points condense in a lower layer with the temperature of boiling point (Kinsara, Demirbas, & Technology, 2016). Finally, the product from this process is liquid gas, gasoline, lubricant, etc. The manufacture of lubricating oil consists of five basic steps (Sequeira, 1992: Sequeira, 1994: Lu & Kaplan, 2008):

- Distillation: Crude oil is first distilled to remove gases, gasoline, naphthas, kerosine, and light gas oil.

- Deasphalting: The vacuum residuum contains recoverable lubricant stock of high viscosity mixed with asphalt and resins. This oil is separated from the asphalt and resins using propane deasphalting, anextractive precipitation process.

- Refining: The deasphalted oil and the distillates usually contain undesirable constituent such as aromatics and naphthenes and these must be removed to yield an oil of high viscosity index and high lubricating quality. Furfural refining is used for elimination of non-ideal components, for example, polycyclic short side chain aromatic hydrocarbons, resin, cycloalkanes, sulfur and nitrogen compounds, from the feedstock oil.

- Dewaxing: Produce a lubricating oil which is not a solid at low temperatures, the wax is removed by solvent dewaxing. A selective hydrocracking process called catalytic dewaxing is used as an alternative to solvent dewaxing and hydrogen finishing for the removal of wax is

removed by solvent dewaxing prior to catalytic dewaxing. Ketone benzene dewaxing is applied for the removal of wax components from the refining oil.

- Finishing: The dewaxed oil is normally hydrofinished or treated with adsorbent clay to meet the color and oxidation stability requirements of products. A severe hydrogen finishing process, hydrorefining, is used to remove large amounts of sulfur and nitrogen and trace impurities in the manufacture of oils or to stabilize base oils produced by hydrocracking. Solvent refining is also used to stabilize hydrocracked base oils.

In the production of lubricants, there are two types of substances that illustrate the danger to living organisms:

- Furfural may be released to the environment during its manufacture. Furfural (2-Furaldehyde). It is a corrosive flammable liquid which is a toxic substance according to the Notification of Department of Industrial Works in 2007, Guideline Safe Storage of Dangerous Goods and Dangerous Substances, which is exported furfural many countries, such as The United States, Dominican Republic, China, South-Africa, Thailand, Indonesia, and Korea (HEDSET, 1997). It is assumed that the use of Furfural greater than 240,000 tonnes per year (CEH, 1994). The lowest measured NOEC (No Observed Effect Concentration) long-term of fish is 0.33 mg/l (Witters, 2005), algae is 2.7 and 31 mg/l (Bringmann & Kühn, 1978) and invertebrates is 1.9 mg/l (Palmer *et al.*, 2005). Human health hazards furfural is classified as a Category 3 carcinogen (R40; limited evidence of a carcinogenic effect).

- Benzene is carcinogen and causes haematotoxicity through its phenolic metabolites that act in concert to produce DNA strand breaks, chromosomal damage, sister chromatid exchange, inhibition of topoisomerase II and damage to mitotic spindle (Rana & Verma, 2005).

Lubricants certain 90% base oil and less than 10% additives after physical mixing constitute the lubricating oil. In part of additive manufacture of lubricating oil that has effects for environment. Most of them are dithiophosphates, overbased calcium sulphonates, succinimidic dispersants, and polymer VI improvers. These can release pollution that is dangerous for environment,such as H2S, HCl, and sludge (Herdan, 1997).

Ashless succinimide dispersants used and manufactured lubricating oil additives are ashless succinimide dispersants. They are used at levels approaching 50 % in all automotive crankcase additive packages. Toxicity of succinimide ashless dispersants are not considered harmful if they swallowed or absorbed through the skin. They are neither eye nor skin irritants, and they do not cause an allergic skin reaction based on guinea-pig sensitization tests (Madanhire & Mbohwa, 2016).

Sulphonates and phenates are important group of additives known as metallic detergents. Sulphonates are used in virtually all types of lubricants, while phenates are used primarily in marine, railroad, and automotive crankcase oils. They are not considered harmful if they swallowed or absorbed through the skin. They are neither eye nor skin irritants. They

do not cause serious systemic effects in sub-chronic toxicity tests (Madanhire & Mbohwa, 2016).

Zinc dialkyldithiophosphates (ZDTPs) are antioxidants and anti-wear additives. Toxicity of Zinc dialkyldithiophosphates are eye irritation potential though not irritating to the skin. It was found out that ZDTPs are mutagenic ("Chapter 2 - Lubrication Chemistry," 2003: Madanhire & Mbohwa, 2016).

Rust inhibitors are used in a variety of industrial and crankcase lubricants. Toxicity of rust inhibitors are not expected to be harmful if they swallowed or absorbed through the skin. They are neither eye nor skin irritants, and they do not cause allergic skin reactions. Rust inhibitors are not mutagenic, and it does not cause serious systemic effects in sub-chronic toxicity tests (Madanhire & Mbohwa, 2016).

Soil degeneration causes by a variety of reasons. But one of the main causes is the contamination of hydrocarbons obtained from the petroleum source and leaks into the environment motive by an accident while transporting, petroleum industry, car repair workshops, environmental smuggling. As mentioned above, can be seen that the problem of soil contaminated with hydrocarbons is coming more widespread (Onuoha et al., 2011). Due to social development and increasing population, people have demand for consuming petroleum products increasingly. For this reason, the chances of hydrocarbon contaminants have increased in the environment. In addition, countries where governments do not have a strict policy to control the smuggling of hydrocarbon waste have widened the amount of hydrocarbon contamination (Ekanem and Ogunjobi, 2017). Waste lubricating oil contain toxic and harmful substances, such as benzene, lead, cadmium, polycyclic aromatic hydrocarbons (PAHs), zinc, arsenic, polychlorinated biphenyls (PCBs) etc., which are hazardous and detrimental to the soil and the surrounding environment (Udonne and Onwuma, 2014). Waste lubricants reduced the pH thereby making the soil more acidic. There is considerable reduction in potassium content in the contaminated soil, as well as increase in carbon contents this being attributed to carbon present in waste oil. An increase in moisture is also noted, as originated from used oil. Thus, the result in adverse effects on the physical and chemical properties of the soil. The physical properties of are altered, when looking at properties like bulk density, capillarity, porosity, and water holding capacity properties of the soil making it less productive (Madanhire & Mbohwa, 2016). The common impacts of lubricants disposed without due care are soil degradation and water contamination resulting. It is regarded that deliberate effort is undertaken to reduce negative environmental impacts of lubricants throughout the value chain from blending to disposal by manufacturers and users.

The problems of contaminated soil have led to the exploration of many solutions that affect the cleaning of contaminated soil. Microorganisms are used to convert harmful substances into non-toxic substances. This is a new technology for treatment. It is known as bioremediation. When treating lubricating oil from the soil, biological methods are preferable a good option to physic-chemical methods because bioremediation is inexpensive equipment, be used easily and environmentally friendly. A combination of treatments, consisting of the application of bioaugmentation and biostimulation. It is evaluated in situ. Bioaugmentation is

the addition of bacteria from other sources for treatment in contaminated soil. Biostimulation is using indigenous bacteria for degradation by adding nutrients, minerals, soil conditioning and aeration to suit the bacterial growth (Odokuma and Dickson, 2003; Ayotamuno *et al.,* 2006). Bacterial growth depends on environmental factors such as pH, temperature, moisture, oxygen, nutrient et cetera. For example, when the bacteria are incubated at different temperatures for 2 days, it is found that bacteria grow at 30-37°C. Higher temperature founds fewer bacteria (Okpokwasili and Okorie, 1988; Mandri and Lin, 2007).

The study of soil microorganisms is both counting and isolated microorganisms from the soil. Bacteria are the most studied group of microorganisms in soil because they are the most abundant in the soil. Soil bacteria count can be done by counting bacteria directly through a microscope. However, it is not as popular as another method, which is to cultivate soil bacteria in a cultured medium that can be seen with the naked eye and then count the bacteria from the colonies. In this way, the number of bacteria is less than reality because the bacteria are diluted. Microorganism culture techniques for example, spread plate technique is a technique in which a 0.1 ml of diluted soil sample is added to the surface of the middle surface of the culture medium. Then, spread the sample over the surface of the culture medium. Then, plate sample is incubated until the microbes grow. The advantage of this technique is that microorganisms do not die from the heat of the culture medium and all cells receive sufficient oxygen. Pour plate technique is a technique commonly used for counting and isolated bacteria from soil samples. 1.0 ml of the diluted sample is added to the liquid medium in the petri dish.

Then, the culture media is set solid and incubated until the microorganisms grow. Then, the colonies are counted in the plate (Younus *et al.,* 2020). The streaking plate technique is commonly used for isolated bacteria. This technique used to isolate a pure strain from a single species of bacteria. The samples can be taken from the resulting colonies and bacteria culture can be grown on a new plate, so that the organism can be identified or tested (Dilmi *et al.,* 2017; Ahda *et al.,* 2018; Younus *et al.,* 2020). The bacterial isolates are recorded for colonial morphology such as margin, color, elevation, texture and shape. In addition, isolated bacteria can also be identified by a variety of methods, such as biochemical. Gram reaction, spore staining and oxidase test et cetera are tested for biochemical characteristics (Okpokwasili and Okorie, 1988; Onuoha *et al.,* 2011; Jayashree *et al.,* 2012; Ekanem and Ogunjobi, 2017).

Therefore, this study aims to isolate some bacteria species from contaminated soil with spent lubricating oil from a car repair workshop and investigate their ability to degrade lubricating oil.

1.2 Objectives

- To isolate some bacteria species from contaminated soil with spent lubricating oil.
- To investigate their ability to degrade lubricating oil.

1.3 Expected Outcome

- To separate bacteria which is effective for degradation of lubricant oil.
- To investigate carbon dioxide by activity of bacteria.

CHAPTER 2

Theory and Literature Review

2.1 Petroleum Formation

Petroleum is a product from anaerobic conversation of organic matter under high temperature and pressure (Balba *et al.,* 1998a). The word ‘petroleum’ derived from the Latin petra and oleum. It means literally rock oil and refers to a mixture of compounds in petroleum products that are all made entirely from hydrogen and carbon, hence the name "hydrocarbon” (Speight, 1999). According to generally accepted theory, petroleum is derived from primitive biomass. Formation of petroleum occurs from hydrocarbon pyrolysis, in a variety of mostly endothermic reactions at high temperature and pressure (Braun & Burnham, 1993). Crude oil that found in the sea is formed by depositing large volumes of zooplankton and algae under prehistoric anoxic conditions (Kvenvolden, 2006). Eventually, over geological time the organic material is compressed between Earth’s mantle under heavy layers of sediment resulting in high heat and pressure. This process causes the organic matter to change. First, into a waxy material known as kerogen, and then more heat, time, and pressure, the kerogen underwent a process called catagenesis, and transformed into hydrocarbons (Braun & Burnham, 1993).

2.2 Classification of Crude Oil

Crude oil according to the United States Environmental Protection Agency (1996) can be classified into the following as discussed:

Class A: Light, Volatile Oils

These oils are often highly fluid, spread rapidly on solid or water surfaces. There is a strong odour, a high evaporation rate, and is usually flammable. They penetrate porous surfaces, such as sand and mud, may be persistent in such a matrix. They do not tend to adhere to surfaces; flushing with water generally removes them. Class A oils may be highly toxic to humans, fish, and others biota. Most refined products and many of the highest quality light crudes can be included in this class.

Class B: Non-sticky oils

These oils have a waxy or oily feel. Class B oils are less toxic and adhere more firmly to surfaces than Class A oils, although they can be removed from surfaces by vigorous flushing. As temperature rises, their tendency to penetrate porous substrates increases and they can be persistent. Evaporation of volatiles may lead to a Class C or D residue. Medium to heavy paraffin-based oils fall into this class.

Class C: Heavy, sticky oil

Class C oils are characteristically viscous, sticky or tarry, and brown or black in appearance. Flushing with water will not readily remove this material from surfaces, but the oil does not readily penetrate porous surfaces. The density of Class C oils may be near that of water. Weathering or evaporation of volatiles may produce solid or tarry Class D. oil. Toxicity is low, but wildlife can be smothered or drowned when it is contaminated. This class includes residual fuel oils and medium to heavy crudes.

Class D: Non-fluid oils

Class D oils are relatively non-toxic, do not penetrate porous substrates, and are usually black or dark brown in colour. When it is heated, Class D oils may melt and coat surfaces making clean up very difficult. Residual oils, heavy crude oils, some high paraffin oils, and some weathered oils fall into this class.

2.3 The Identity and Origin of Lubricating Oil

Lubricating oil is products from crude oil distillation industry, it is known as fractional distillation processes. Lubricating oil is boiled range 405-515°C at 1 atm, rang of carbon 26-38 molecule, 10% of Class B: Non-sticky oils original petroleum ("Chapter 16 Petroleum Lubricating Oils," 1985). The chemical composition of lubricating oil, as with all other petroleum products, varies with the crude-oil source and the additives present. The additives are shown in Fig 1 (Hewstone, 1994).

Major types of chemical additives used in lubricant formulations

Component	Chlorine content (%)
Zinc alkaryl dithiophosphate	< 0.002
Zinc alkyl dithiophosphate	< 0.002
Calcium long-chain alkaryl sulphonate	0.26
Calcium long-chain alkaryl sulphonate	0.28
Calcium long-chain alkyl phenate sulphide	< 0.002
Calcium long-chain alkyl salicylate	< 0.002
Magnesium long-chain alkaryl sulphonate	0.01
Polyolefin amide alkeneamine borate	0.13
Polyolefin amine	0.20
Alkyl sulphide	0.25
Olefin/alkyl ester copolymer	< 0.002
Poly alkyl methacrylate	< 0.002
Polyolefin	< 0.002
Polyolefin ester	0.14
Long-chain chloralkane	35
Hydrocarbon polymer	0.79
Gear oil additive	1.72
Commercial oil	0.019

The limit of detection in the tests was 0.002%.

Figure 1 Major types of chemical additives used in lubricant formulations (Hewstone, 1994).

2.4 Environmental and Health Concern with Lubricating Oil

Lubricating oil can change the physical and chemical composition of the soil because lubricant contamination affects the distribution of organic matter and nutrients in the soil which affects the growth of soil microorganisms. Lubricating oil is also responsible for the depletion of oxygen and moisture because the film of the lubricant is difficult for passing through oxygen and moisture. It probably causes the death of the aerobic microorganisms and affect soil fertility (Nowak *et al.*, 2019; Younus *et al.*, 2020). In addition, lubricating oil contaminate the water, problem is similar of soil contamination. The film of lubricating oil on the water probably causes disturbances in the oxygen exchange between the water and the atmosphere, it also reduces the access of light (Nowak *et al.*, 2019). Lubricating oil compound is carcinogens and mutagens substances in humans and animals. Humans exposed to long-term show higher incidence of cancer. Additionally, there may be allergic reactions, irritation, headaches, and fatigue (Ahda *et al.*, 2018; Nowak *et al.*, 2019; Younus *et al.*, 2020).

2.5 Exposure Pathway

The general people may touch with lubricating oil by touching with contaminated soil through skin touch, accidental ingestion or inhalation of dust (Mangas *et al.*, 2014). For example, when lubricating oil is released into water, contact can occur from ingestion and skin contact with contaminated water. In addition, humans, animals, and plants get from food chain (Odjegba and Sadiq, 2002).

2.6 Biodegradation of Lubricating Oil

Biodegradation is the chemical breakdown of materials by the physiological environment or breaking down of complex, and possibly toxic material into simple and common forms. Organic materials can be degraded aerobically with oxygen, or without oxygen. A term related to biodegradation is biomineralisation, in which organic matter is converted into minerals. Biosurfactant, an extracellular surfactant secreted by microorganisms, enhances the process of biodegradation (Diaz, 2008; Aluyor & Ori-Jesu, 2009).

Biodegradation in other terms is a treatment process whereby contaminants or pollutants are metabolized into non-toxic or less toxic compounds by microorganisms naturally existing in a given environment. Microorganisms can utilize many of the petroleum hydrocarbon constituents as a source of carbon and energy producing carbon dioxide and water as by-products. Once all of the contaminants have been consumed by microorganisms, the microbial population becomes dormant or dies out. Biodegradation can take place under aerobic or anaerobic conditions in the presence of other suitable electron acceptors such as nitrate, sulfate, or carbonate (Balba *et al.*, 1998a).

In biodegradation, lubricating oil is not directly degradable because it is insoluble in water and diverse additives (Nowak *et al.*, 2019). Biodegradability parameters that are considered an indicator of the consumption of the test substance by microorganisms, various

biodegradability testing methods are available as measured by the amount of carbon dioxide or methane for non-use oxygen case (Aluyor & Ori-Jesu, 2009).

Factors affecting the microbial degradation, for example, moisture is related to the water content of the soil, which microorganisms need to use water as a solvent and osmosis process. Salt concentration of soil affects the osmotic pressure of microbial cells. The cells of microorganisms do not like salinity are inhibited, while the salinity-resistant microbial cells can survive and multiply slightly. The salinity-like microbial cells can grow and multiply well (Imhoff, 1993). Low salinity microorganisms grow in 1-3% salt medium, while high salt microorganisms grow in 15-32% salt medium (Kushner, 1985). Oxygen stimulates the production of carbon dioxide and the biodegradation of oil. The temperature is the most important parameter in the biodegradation of lubricating oils at 25°C, 50-80% of the initial oil is biodegraded (Vazquez-Duhalt, 1989). At 25°C, it is not the best temperature for microbial growth. The optimal temperature range for growth is 30-37°C (Okpokwasili & Okorie, 1988; Mandri and Lin, 2007).

Current research reveals that in aquatic and terrestrial environments microorganisms are the chief agents for the biodegradation of molecules of petroleum hydrocarbons (Swanell & Head, 1994; Balba *et al.*, 1998a). Bacteria and yeast appear to be the dominant degraders in aquatic ecosystems, while fungi and bacteria are the main degraders in soil environments (Cooney & Summers, 1976; Hanson *et al.*, 1997; Balba *et al.*, 1998b).

2.7 Oil Degrading Microbes

Some microorganisms have the astonishing, naturally occurring, microbial catabolic diversity to degrade, transform or accumulate a huge range of compounds including hydrocarbons (oil), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), pharmaceutical substances, radionuclides and metals (Diaz, 2008). It is well established fact that no single species of microorganisms can completely degrade any particular oil and this idea has been widely accepted by the scientific community (Colwell & Walker, 1977; Balba *et al.*, 1998a).

The most common genera known to be responsible for oil degradation or breakdown comprise mainly *Nocardia*, *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Micrococcus*, *Arthrobacter*, *Corynebacterium*, *Achromobacter*, *Rhodococcus*, *Alcaligenes*, *Mycobacterium*, *Bacillus*, *Aspergillus*, *Mucor*, *Fusarium*, *Penicillium*, *Rhodotorula*, *Candida* and *Sporobolomyces* spp. (Atlas, 1981; Bossert & Bartha, 1984; Okpokwasili & Okorie, 1988; Atlas & Bartha, 1992; Sarkhoh *et al.*, 1990; Balba *et al.*, 1998b; Onuoha *et al.*, 2011; Jayashree *et al.*, 2012; Jesubunmi & Healthcare, 2014; Younus *et al.*, 2020).

In 1984, found out shorter alkanes of intermediate length ($C_{10} – C_{20}$) are the preferred substrates and tend to be most readily degradable unlike branched chain alkanes which are degraded more slower than the corresponding normal alkanes (Singer & Finnerty, 1984).

Longer chain alkanes known as waxes ($C_{20} – C_{40}$) are hydrophobic solids and consequently are difficult to degrade due to their poor water solubility and bioavailability (Bartha, 1986).

2.8 Pathways of Petroleum Hydrocarbon Degradation

Microorganisms gain energy by catalyzing energy-producing chemical reactions. The type of chemical reaction is called oxidation-reduction reaction and involves the transfer of electrons away from the contaminant. In the process, the organic contaminant is oxidized, the technical term for losing electrons. Correspondingly, the chemical that gains the electrons is reduced. The contaminant is called the electron donor, while the electron recipient is called the electron acceptor. The energy gained from these electron transfers is 'invested', along with some electrons and carbon from the contaminant, to produce more cells (Nyer, 1993).

In 1992, found out three main energy-yielding oxidation-reduction processes by which non-photosynthetic microorganisms can break down hydrocarbons to obtain energy namely through; fermentation, aerobic respiration and anaerobic respiration. The amount of energy available depends on the metabolic pathway utilized and the available carbon source (Riser-Roberts, 1992).

The process of degrading organic compounds with oxygen involved is called aerobic respiration. In aerobic respiration, microbes use oxygen to oxidize part of the carbon in the contaminant (hydrocarbon) to carbon dioxide, with the rest of the carbon used to produce new cell mass. Thus, the major by-products of aerobic respiration are carbon dioxide, water, and an increased population of microorganisms (Freeze & Cherry, 1979; Levin & Gealt, 1993). In the process of degradation of organic compounds without oxygen is called anaerobic respiration. In anaerobic respiration, nitrate, sulfate, metals, such as iron and manganese, or even carbon dioxide can play the role of oxygen, accepting electrons from the contaminant. Thus, anaerobic respiration uses inorganic chemicals as electron acceptors. In addition, to new cell matter, the by-products of anaerobic respiration may include nitrogen gas, hydrogen sulfide, reduced forms of metals, and methane, depending on the electron acceptor (Freeze & Cherry, 1979; Levin & Gealt, 1993). Aerobic biodegradation of hydrocarbons compared to the other degradation pathways is faster than fermentation and anaerobic biodegradation. Anaerobic biodegradation on the other hand could be much less expensive than the more commonly considered aerobic approach, which is costly and energy intensive due to the need for vigorous agitation and aeration in order to introduce sufficient quantities of oxygen (Aitken, 2004).

2.9 Principle and chemistry of aerobic degradation

The most rapid and complete degradation of majority of organic pollutants is brought about under aerobic conditions. Figure 2 shows the main principle of aerobic degradation of hydrocarbons. The initial intracellular attack of organic pollutants is an oxidative process and the activation as well as incorporation of oxygen is the enzymatic key reaction which is catalyzed by oxygenases and peroxidases. This attack normally results in the addition of hydroxide to the alkane (hydrocarbon) to form alcohol which is subsequently oxidized into

aldehyde and finally into fatty acids. The addition of oxygen to hydrocarbon compounds makes them more polar and thus more soluble. So, peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, for example, the tricarboxylic acid cycle. Biosynthesis of cell biomass occurs from the central precursor metabolites, for example, acetyl-CoA, succinate, and pyruvate. Sugars required for various biosynthesis and growth are synthesized through gluconeogenesis (Das & Chandran, 2011; Olajire *et al.,* 2014).

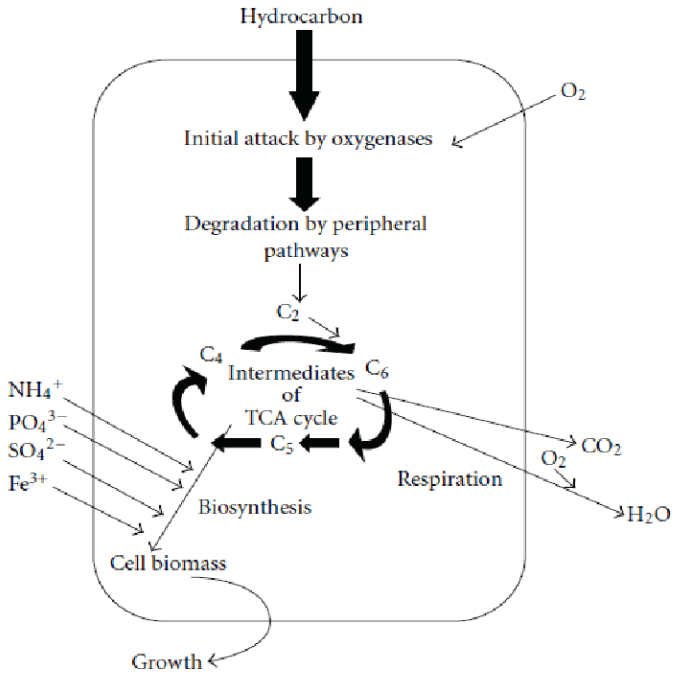

Figure 2 Main principle of aerobic degradation of hydrocarbons by microorganisms (Das & Chandran, 2011).

2.10 Bioremediation

Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities (Mueller *et al.,* 1996).

By definition, bioremediation is the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms. It is uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health or the environment. The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Contaminant compounds are transformed by living organisms through reactions that take place as a part of their metabolic processes (Vidali, 2001).

Bioremediation has limitations. Some contaminants, such as chlorinated organic or high aromatic hydrocarbons, are resistant to microbial attack. They are degraded either slowly or not at all, hence it is not easy to predict the rates of clean-up for a bioremediation exercise;

there are no rules to predict if a contaminant can be degraded. Bioremediation techniques are typically more economical than traditional methods, such as incineration, and some pollutants can be treated on site.Thus, reducing exposure risks for clean-up personnel, or potentially wider exposure as a result of transportation accidents. Since, bioremediation is based on natural attenuation the public considers more acceptable than other technologies (Vidali, 2001).

In 2003, bioremediation was applied to the treatment in-situ of crude oil contamination tropical rain forest soil performing combination treatment with biostimulation and bioaugmentation into application by Odokuma and Dickson. They were found that if bioaugmentation was used with indigenous bacteria, 73% of crude oil was degraded. While using biostimulation with bioaugmentation with indigenous bacteria, 88% of crude oil was degraded (Odokuma and Dickson, 2003). Three years later, bioremediation researching was applied biostimulation by adding fertilizers and oxygen in-situ tropical rain forest soil with crude oil. It is found between 75 and 200 gm of fertilizer per 0.16 m^2 to the fastest biodegradation (Ayotamuno *et al.*, 2006).

2.11 Factors Affecting Bioremediation

Bioavailability is extremely important to biodegradation of organic pollutants. It is frequently observed that the rate of removal of compounds from soils is very low, even though the compounds are biodegradable. The three main classes of hydrocarbons (aliphatic, alicyclic, and aromatic hydrocarbons) vary in their biodegradability according to size and solubility (Atlas & Unterman, 1995).

Many contaminated sites are characterized by poor nutrient concentrations (i.e., low levels of nitrogen and phosphorus), elevated or low temperatures, and a diverse range of contaminants, such as heavy metals, which can influence the process of bioremediation by inhibiting the growth of the pollutant-degrading microorganisms. Therefore, the efficacy of pollutant transformation and potential for bioremediation. Indeed, bioremediation treatments are often designed to overcome these limitations which may contribute to environmental persistence of particular importance are pH, redox potential, supply of oxygen, moisture, temperature, inorganic nutrients, cation-exchange capacity, pollutant bioavailability, and soil porosity (Atlas & Unterman, 1995).

The most important factor limiting rates of biodegradation in the environment is the availability of molecular oxygen. The initial enzymes of aerobic attack on hydrocarbons are oxygenases, which have an absolute requirement for molecular oxygen. Delivering air or oxygen to contaminated soils can be difficult for a number of reasons: the soil porosity may not be favorable. Therefore, mass transfer from the gas phase to the aqueous phase will be limited. Also, the relatively low solubility of oxygen in water is a primary limiting factor (Atlas & Unterman, 1995).

Thus, the factor that affects to bioremediation are the existence of a microbial population capable of degrading the pollutants; the availability of contaminants to the microbial

population; the environment factors (type of soil, temperature, pH, the presence of oxygen or other electron acceptors, and nutrients) (Vidali, 2001).

2.12 In Situ Bioremediation

This technique is generally the most desirable options due to lower cost and less disturbance since they provide the treatment in place avoiding excavation and transport of contaminants treatment is limited by the depth of the soil that can be effectively treated. In many soils effective oxygen diffusion for desirable rates of bioremediation extend to a range of only a few centimetres to about 30 cm into the soil, although depths of 60 cm and greater have been effectively treated in some cases. Among the several available in situ technologies, the following can be mentioned:

Bioventing is the most common in situ treatment and involves supplying air and nutrients through wells to contaminated soil to stimulate the indigenous bacteria. Bioventing employs low air flow rates and provides only the amount of oxygen necessary for the biodegradation while minimizing volatilization and release of contaminants to the atmosphere (Fig 3). It works for simple hydrocarbons and can be used where the contamination is deep under the surface (Vidali, 2001). Bioventing is highly efficient especially for hydrocarbon-contaminated sites (Zouboulis & Moussas, 2011).

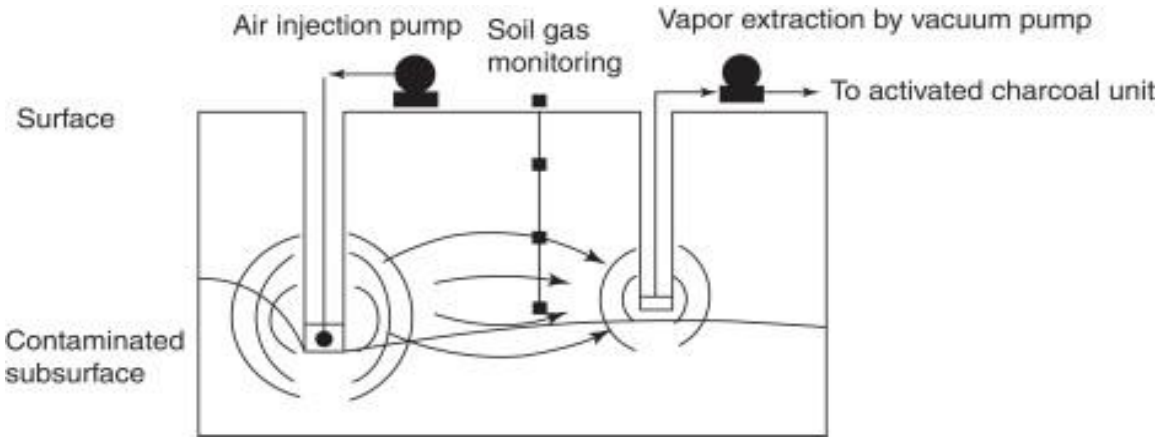

Figure 3 Application of bioventing technology (Zouboulis & Moussas, 2011).

Biosparging involves the injection of air under pressure below the water table to increase groundwater oxygen concentrations and enhance the rate of biological degradation of contaminants by naturally occurring bacteria. Biosparging increases the mixing in the saturated zone and thereby increases the contact between soil and groundwater. The ease and low cost of installing small-diameter air injection points allows considerable flexibility in the design and construction of the system (Vidali, 2001).

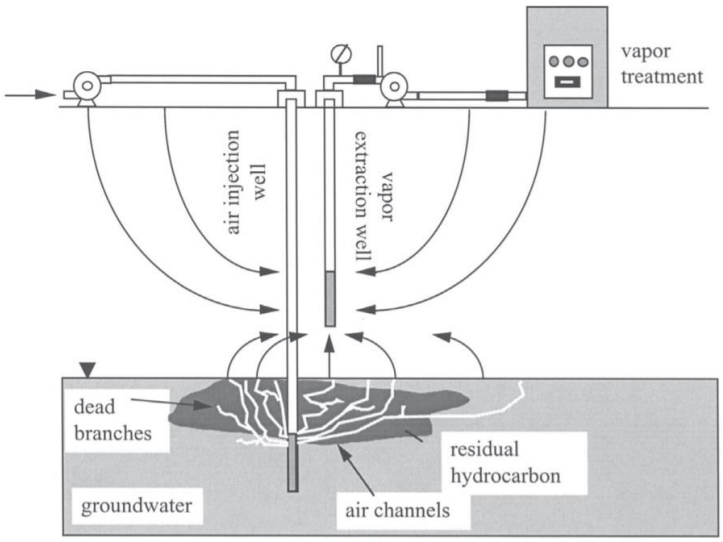

Figure 4 Schematic diagram of a simplistic in situ air sparging system combined with soil vapor extraction (Johnson *et al.*, 2001).

Bioaugmentation involves the addition of microorganisms indigenous or exogenous to the contaminated sites. Two factors limit the use of added microbial cultures in a land treatment unit: 1) nonindigenous cultures rarely compete well enough with an indigenous population to develop and sustain useful population levels and 2) most soils with long-term exposure to biodegradable waste have indigenous microorganisms that are effective degrades if the land treatment unit is well managed (Vidali, 2001).

2.13 Ex Situ Bioremediation

This techniques involve the excavation or removal of contaminated soil from the site to another location for further treatment. The most important and common techniques falling in this category include:

Landfarming is a simple technique in which contaminated soil is excavated and spread over a prepared bed and periodically tilled until pollutants are degraded. The goal is to stimulate indigenous biodegradative microorganisms and facilitate their aerobic degradation of contaminants. In general, the practice is limited to the treatment of superficial 10–35 cm of soil. Since landfarming has the potential to reduce monitoring and maintenance costs, as well as clean-up liabilities, it has received much attention as a disposal alternative (Vidali, 2001). The advantages of this technology include mainly: (1) a significant reduction of surface area required for treatment, (2) reduced remediation time due to improved design, and (3) ease of applied treatment (Zouboulis & Moussas, 2011).

Composting is a technique that involves combining contaminated soil with nonhazardous organic amendants ,such as manure or agricultural wastes. The presence of these organic materials supports the development of a rich microbial population and elevated temperature characteristic of composting (Vidali, 2001). The major advantage of composting,

when compared to land farming, is that it enables the improved control and optimization of the process, so that the rate and extent of microbial activity become significantly better than those in land farming (Zouboulis & Moussas, 2011).

Bioreactors are used for the ex situ bioremediation treatment of contaminated soil, or of water coming from a contaminated plume. This technology offers through the control of critical parameters, such as microbial population, nutrients, pH, and moisture, the near-perfect environment for biodegradation. However, there arealso some inevitable disadvantages: (1) excavation of soil and pumping of groundwater are necessary, (2) during the application of treatment a certain amount of sludge (treated material plus biomass) and a volume of gases (e.g., carbon dioxide, methane, hydrogen sulfide) are produced, considered eventually as secondary pollutants, which require further treatment, thus considerably increasing the cost (Zouboulis & Moussas, 2011).

2.14 Advantages of Bioremediation

- Bioremediation is a treatment process that people accept because it is a natural process that does not affect for the creatures and environment.
- Residue after treatment by Bioremediation process is not dangerous.
- Bioremediation can change dangerous toxin to be less.
- Bioremediation is a completely degradation method without the use of medium. For example, from land to water or air, the complete destruction of target pollutants is possible.
- Bioremediation can often be carried out on site. This also eliminates the need to transport quantities of waste off site and the potential threats to human health and the environment that can arise during transportation.
- The operation cost of Bioremediation is lower than other treatment processes (Vidali, 2001).

2.15 Disadvantages of Bioremediation

- Bioremediation is limited to compounds that are biodegradable.
- Bioremediation often takes longer than other treatment options.
- Bioremediation is a process that depend on factors relating to microorganisms and the environment. In other word, the presence of metabolically capable microbial populations, suitable environmental growth conditions, and appropriate levels of nutrients and contaminants.

- It is difficult to extrapolate from bench and pilot-scale studies to full-scale field operations.
- There is no accepted definition of “clean”, evaluating performance of bioremediation is difficult, and there are no acceptable endpoints for bioremediation treatments.
- There are some concerns that the products of biodegradation may be more persistent or toxic than the parent compounds.
- Research is needed to develop and engineer bioremediation technologies that are appropriate for sites with complex mixtures of contaminants that are not evenly dispersed in the environment. Contaminants may be present as solids, liquids, and gases (Vidali, 2001).

CHAPTER 3

Materials and Methods

3.1 Chemicals

- Contaminated soil with spent lubricating oil from a car repair workshop in Chiang Mai.
- Bushnell-Hass medium (BHM) consisted of $MgSo_4.7H_2O$ 0.2 gm, K_2HPO_4 1 gm, $FeCl_3$ 0.05 gm, NH_4NO_3 1 gm, $CaCl_2$ 0.02gm in 1 L of distilled water.
- Spent lubricating oil from a car repair workshop in Bangkok.
- Trypticase soy broth (TSB) and Agar
- Ethyl Alcohol 75% and 95%
- Crystal Violet for Gram Stain
- Safranin O for Gram Stain
- Iodine for Gram Strain
- Sodium Hydroxide (NaOH)
- Hydrochloric Acid (HCl)
- Barium Chloride ($BaCl_2$)
- Phenolphthalein
- Distilled Water
- Bushnell-Hass medium (BHM)

3.2 Equipment

- Needle
- Dropper
- Petri Dish
- Autoclave
- Glass Tube
- Glass Slide
- Clean Bench
- Stirring Rod
- Glass Funnel
- Silicone Tube
- Vortex Mixer
- Burette Clamp
- Alcohol Lamp
- Glass Spreader
- Burette 50.0 ml
- Silicone Stopper
- Colony Counter
- Inoculation Loop
- Flask 100 and 250 ml
- Test Tube 15 and 20 ml
- Beaker 100, 250, and 500 ml
- Glass Pipettes 0.1, 1.0, 10.0 ml
- Incubator and Shaking Incubator
- Laboratory Chemical Fume Hood
- Stereo Microscope (HTT, 1967) and Digital Camera (Nikon, Digital Sight DS-Fi1)
- Glass Measuring Cylinder 100 and 500 ml
- Light Microscopes (OLYPUS U-SPT)
- Scientific Balances Scale

3.3 Methods

3.3.1 Sampling

A sample of contaminated soil with spent lubricating oil belong to car repair workshop in Chiang Mai. Area with heavy oil spot is selected and the sample is collected at 0-5 cm depth adapt from Younus *et al.*, 2020.

3.3.2 Bacteria Capable to Degradation of Lubricating oil

Ten grams of contaminated soil with spent lubricating oil is added to 90.0 ml of sterile distilled water. Then, 10.0 ml is added to flak containing BHM 100 ml and spent lubricating oil 1.0 ml repeat 3 more times. When complete 3 rounds, 1.0 ml adds to obtain dilution 10^{-1} to 10^{-7} of distilled water. Then, 0.1 ml spread on plates (spread plate technique) containing TSA (TSB 30 gm L^{-1} + Agar 18 gm L^{-1}) and 0.05 ml or 1 dropper of sterile spent lubricating oil by autoclave 121°C under 15 lb/ng for 15 min. When plate is spread completely, then it is incubated at 37°C for 24-48 hours. After that, the colonies are counted and recorded morphology adapting from Younus *et al.*, 2020.

3.3.3 Isolation of Bacteria capable to Degradation of Lubricating oil

When colonies are counted completely and recorded morphology, then the inoculation loop (streaking plate technique) used randomly selected 4 types of bacteria to spread out bacteria on new TSA to purify, then incubated at 37°C for 24 - 48 hours repeat 3 more times. Then, a colony is leaded to increase the number of bacteria in flask containing TSB 100 ml. After that, it is incubated at 37°C for 24-48 hours. Whereat, 1.0 ml each flake leaded to obtain dilution 10^{-1} to 10^{-7} of distilled water. Then, 0.1 ml spread on plates (spread plate technique) containing TSA (TSB 30 gm L^{-1} + Agar 18 gm L^{-1}). After that, the plates are incubated at 37°C for 24-48 hours. Then, the colonies are counted and recorded morphology.

3.3.4 Gram Staining

The loop from sterilized by burning spread out a colony from each plate and then leads to spread out on prepared glass slide. After that, drop crystal violet leaved 60 seconds and then washing by distilled water. Then, add gram's iodine, wait for 60 seconds, after that rinse with distilled water. Next, washing by ethyl alcohol 95% on glass slide until return blue, then rinse with distilled water immediately. After that, add safranin O and wait 45 seconds, then rinse and dry. Already taken to light microscope. Observe the results and take notes.

3.3.5 Clear Zone

The plate containing TSA is spread spent lubricating oil 0.1 mL over it and the plate tabulates 2x2 for each bacterium. The needle from sterilized by burning spread out a colony from each plate. After that, spread out on prepared plate (spot plate technique). Then, incubated at 37°C for 24 hours adapt from Burd & Ward, 1996. Observe the results and take notes.

3.3.6 Measurement of Degradation in Lubricating oil

Ten milliliter of culture media was added to flask containing BHM 100 mL and 1.0 mL of spent lubricating oil and connect the glass tube from the culture flask to the prepared flask 100 ml of sodium hydroxide with concentration 1.0 mol L^{-1}. After that, incubated at 37°C /100 rpm for 14 days with control (BHM media 100 mL+ 1.0 mL motor oil only). Then, complete 14 days, leaded each flask of NaOH to add 3.0 mL of $BaCl_2$ (0.5 mol L^{-1}). Several drops of phenolphthalein indicator are added, and the solution titrated against standardized HCl (0.5 mol L^{-1}) adapt from Doran & Zander, 2012. Observe the results and take notes.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Colonies and Morphology of Bacteria Capable to Degradation of Lubricating oil

One concentration was repeated three times since 10^{-1}-10^{-7}. The colonies can count when concentration 10^{-7}. It was found at 24 hours to be able to count two plates (table 1). So, there was the same amounts of bacteria $1.17x10^{9}$ CFU/ml at 24 hours. After completely 48 hours found that number of colonies to increase and size of colonies were bigger (table 2 and figure 5). So, there was the same amounts of bacteria $2.02x10^{9}$ CFU/ml at 48 hours. In fact, black bacterial colonies could not actually be formed, described in detail discussion.

4.2 Result of Randomly Selected Bacterial Gram Staining

Four colonies were selected from plate passed spread before. After purification, all bacteria were stained gram. Four bacteria were found to be gram-negative (figure 6).

4.3 Clear Zone of Four Bacteria

Four bacteria occur clear zone around of colonies (figure 7). From observing found SW2 had clear zone larger than other. SW3 had clear zone least.

4.4 Colonies and Morphology of Four Bacteria Before Measurement of Degradation in Lubricating oil

After purification and increasing of bacteria, four bacteria known and estimated number of bacteria before being tested for biodegradation of lubricants. 1.0 ml for each both to dilution 10^{-1} to 10^{-7}. Then, leaded to spread plates (spread plate technique) incubating at 37°C for 24 hours. Colonies and morphology include CFU/ml of four bacteria shows table 3 to table 6.

4.5 Measurement of Degradation in Lubricating oil From Carbon Dioxide (CO_2)

The ability of bacteria to degrade hydrocarbons of lubricating oil was investigated using titration. Found out SW1 had most carbon dioxide and SW4 had fewest carbon dioxide (figure 13). In 2012, Doran & Zander conducted an experiment to determine the concentration of carbon dioxide from microbial activity for a period of 3-4 weeks (figure 14).

4.6 Discussion

From clause 4.1, when colonies to stereo microscope, the black colonies are lubricating oil glazed on the surface of the colony. It is not colonies because when loop spread out a black colony leads to new TSA without spent lubricating oil on surface of nutrient. Found out color of a black colony is opaques from figure 6 of SW3 and figure 11 of SW3. However, *Salmonella* spp. are black colonies (figure 12) because thiosulfate and ferric citrate permit detection of hydrogen sulfide by the production of colonies with black centers refer from www.microbiologyinfo.com. From several studies have not yet clearly identified the hydrocarbons biodegradability of *Salmonella* spp. (Atlas, 1981; Bossert & Bartha, 1984; Okpokwasili & Okorie, 1988; Atlas & Bartha, 1992; Sarkhoh *et al.*, 1990; Balba *et al.*, 1998b; Onuoha *et al.*, 2011; Jayashree *et al.*, 2012; Jesubunmi & Healthcare, 2014; Younus *et al.*, 2020). After the bacterial colonization was carried out, the stereo microscope found the activity of the bacteria secreting enzymes to break down the lubricant droplet (figure 13). There are a diameter that 7.89 mm to 8.98 mm.

To find out the number of CFU/ ml in the original sample, the number of colony forming units on the countable plate is multiplied by 1/ Final Dilution Factor (FDF). This takes into account all of the dilution of the original sample. Clear zone of colonies bacteria can prove that bacteria can degrade lubricating oil. It can be applied to find the degradation ability of bacteria. The degradation measurements in lubricants can be titrated to determine the carbon dioxide produced by bacterial degradation activity adapting from Doran & Zander, 2012. The process degradation measurements to determine carbon dioxide can be explained by the following equation (figure 16):

$$2NaCl + CO_2 \longrightarrow Na_2CO_3 + H_2O \qquad (1)$$

$$Na_2CO_3 + BaCl_2 \longrightarrow BaCO_3 + 2NaCl \qquad (2)$$

$$BaCO_3 + 2HCl \longrightarrow BaCl_2 + H_2O + CO_2 \qquad (3)$$

In equation 2, add $BaCl_2$ in flask contain Na_2CO_3 occur white precipitate (figure 17). After that, when the previous drop of phenolphthalein was added to the solution, it was found that the solution's color changed from clear to pink. After about 3-5 minutes, the solution returned to its clear color. Then, titrate the solution with HCl ($0.5 molL^{-1}$) until the color of the solution turns pink known as “End Point” (figure 18). From equation 3 can be calculated for carbon dioxide (figure 15). However, compare SW3 and SW4 found that SW3 has $4.3x10^6$ CUF/ml, SW4 has $5.1x10^7$ CUF/ml, but amount of carbon dioxide from the activity of SW4 less than SW3. SW3 has a greater ability to degrade hydrocarbons, although in smaller quantities. According to Doran & Zander's experiments, although it took a longer time to decompose microorganisms, but the resulting CO_2 concentration was lower. This probably to the ability of microorganisms to digest different hydrocarbon compounds (figure13&14).

Table 1 Colonies and Morphology at 24 hours

No. of Plate	Whole	Edge	Elevation	Texture	Colour	Number of Colonies
1	Circular	Entire	Convex	Smooth	Opaque	106
	Circular	Entire	Convex	Smooth	Black	7
	Total of Colonies					113
No. of Plate	**Whole**	**Edge**	**Elevation**	**Texture**	**Colour**	**Number of Colonies**
3	Circular	Entire	Convex	Smooth	Opaque	120
	Circular	Entire	Convex	Smooth	White	1
	Total of Colonies					121

Table 2 Colonies and Morphology at 48 hours

No. of Plate	Whole	Edge	Elevation	Texture	Colour	Number of Colonies
1	Circular	Entire	Convex	Smooth	Opaque	105
	Circular	Entire	Convex	Smooth	White	1
	Circular	Entire	Convex	Smooth	Black	134
	Total of Colonies					240
No. of Plate	**Whole**	**Edge**	**Elevation**	**Texture**	**Colour**	**Number of Colonies**
3	Circular	Entire	Convex	Smooth	Opaque	105
	Circular	Entire	Convex	Smooth	Black	58
	Total of Colonies					163

Table 3 Shows colonies and morphology at 24 hours concentration 10^{-7} of SW1

No. of Plate	Whole	Edge	Elevation	Texture	Colour	Number of Colonies
2	Circular	Entire	Convex	Smooth	Opaque	65
	Circular	Entire	Convex	Smooth	White	1
	Total of Colonies					66
3	Circular	Entire	Convex	Smooth	Opaque	80
	Circular	Entire	Convex	Smooth	White	3
	Total of Colonies					83
Average						74.5
CFU/ml of SW1						$7.45x10^{8}$

Table 4 Shows colonies and morphology at 24 hours concentration 10^{-6} and 10^{-7} of SW2

No. of Plate (10^{-6})	**Whole**	**Edge**	**Elevation**	**Texture**	**Colour**	**Number of Colonies**
2	Circular	Entire	Convex	Smooth	Opaque	276
	Circular	Entire	Convex	Smooth	White	1
	Total of Colonies					277
CFU/ml of SW2 concentration 10^{-7}						$2.77x10^{8}$
No. of Plate (10^{-7})	**Whole**	**Edge**	**Elevation**	**Texture**	**Colour**	**Number of Colonies**
2	Circular	Entire	Convex	Smooth	Opaque	42
	Total of Colonies					42
CFU/ml of SW2 concentration 10^{-7}						$4.2x10^{8}$
Average (CFU/ml)						$3.485\ x10^{8}$

Table 5 Shows colonies and morphology at 24 hours concentration 10^{-5} of SW3

No. of Plate	**Whole**	**Edge**	**Elevation**	**Texture**	**Colour**	**Number of Colonies**
3	Circular	Entire	Convex	Smooth	Opaque	43
	Total of Colonies					43
CFU/ml of SW3						$4.3x10^{6}$

Table 6 Shows colonies and morphology at 24 hours concentration 10^{-6} of SW3

No. of Plate	**Whole**	**Edge**	**Elevation**	**Texture**	**Colour**	**Number of Colonies**
1	Circular	Entire	Convex	Smooth	Opaque	51
	Total of Colonies					51
CFU/ml of SW4						$5.1x10^{7}$

Table 7 The amount of carbon dioxide from the activity of SW1

SW1	**Begin**	**Stop**	**Difference**
1	0.00	9.60	9.60
2	9.60	19.30	9.70
3	19.30	28.90	9.60
Average			9.63
Carbon dioxide content (gm/ml)			0.0106
Carbon dioxide concentration (mmol/L)			240.9

Table 8 The amount of carbon dioxide from the activity of SW2

SW2	**Begin**	**Stop**	**Difference**
1	0.00	8.50	8.50
2	8.50	17.20	8.70
3	17.20	26.30	9.10
Average			8.76
Carbon dioxide content (gm/ml)			0.0096
Carbon dioxide concentration (mmol/L)			218.1

Table 9 The amount of carbon dioxide from the activity of SW3

SW3	Begin	Stop	Difference
1	0.00	8.10	8.10
2	8.10	15.90	7.80
3	15.90	23.40	7.50
Average			7.80
Carbon dioxide content (gm/ml)			0.0086
Carbon dioxide concentration (mmol/L)			195.4

Table 10 The amount of carbon dioxide from the activity of SW4

SW4	Begin	Stop	Difference
1	0.00	7.80	7.80
2	7.80	15.30	7.50
3	15.30	22.60	7.30
Average			7.53
Carbon dioxide content (gm/ml)			0.0080
Carbon dioxide concentration (mmol/L)			181.8

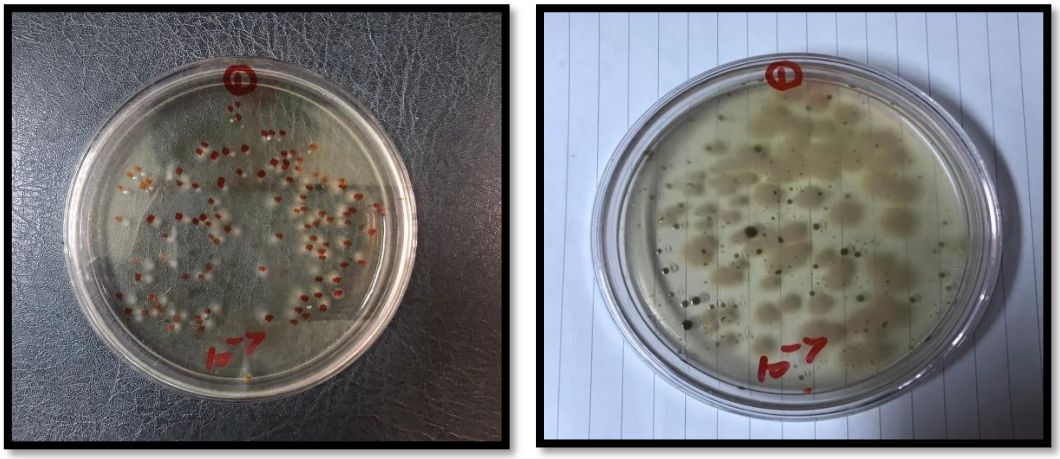

Figure 5 Compare quantity and size of colonies, on the left at 24 h. and on the right at 48 h.

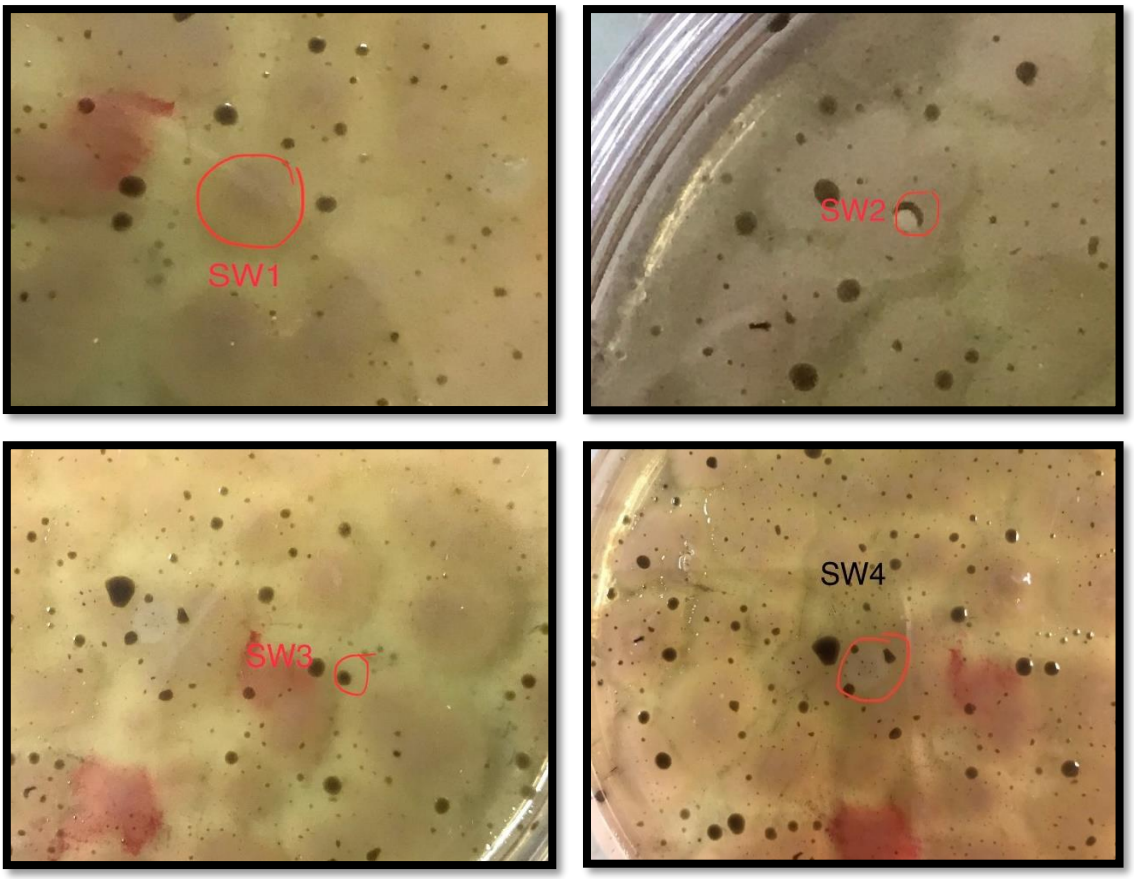

Figure 6 Colonies of bacteria randomly are selected.

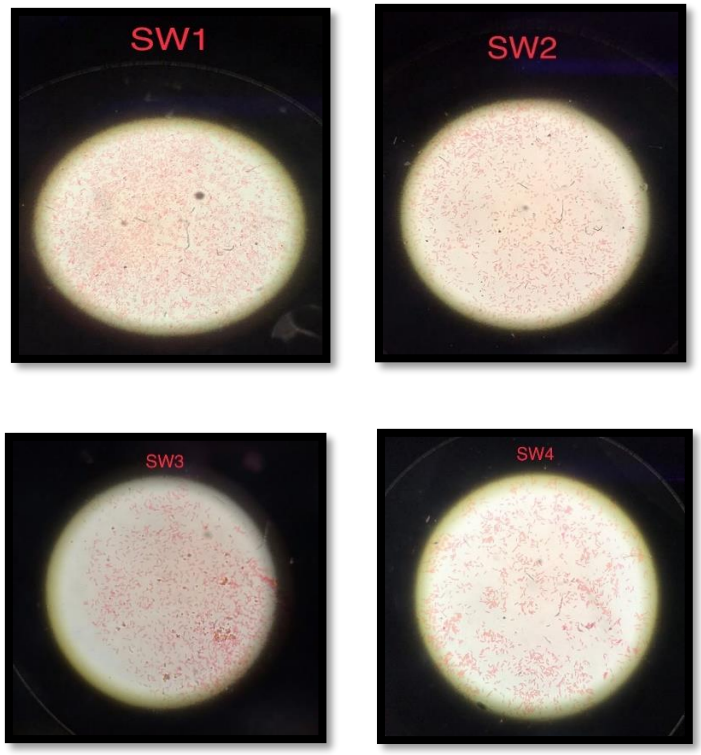

Figure 7 Color gram staining of four bacteria

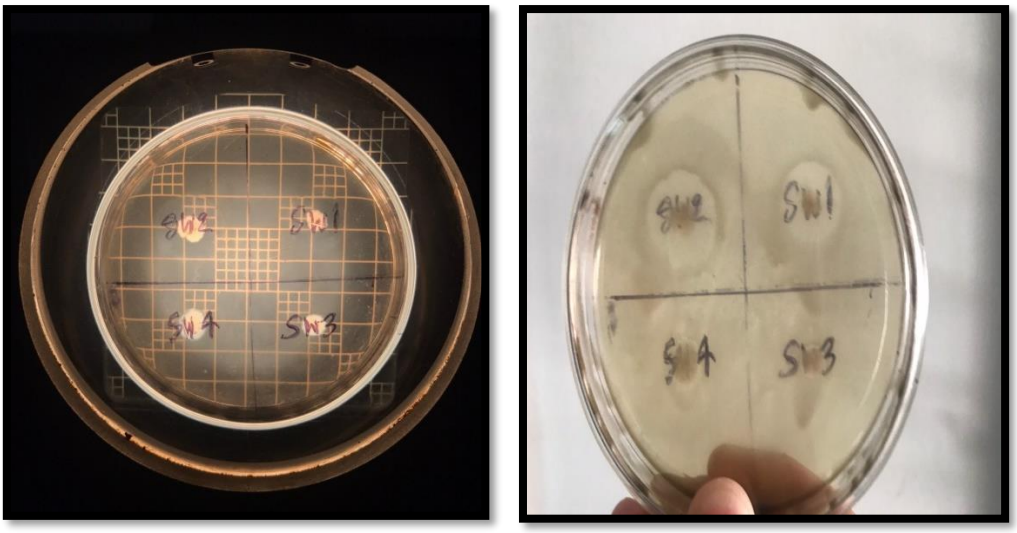

Figure 8 Clear zone of four bacteria

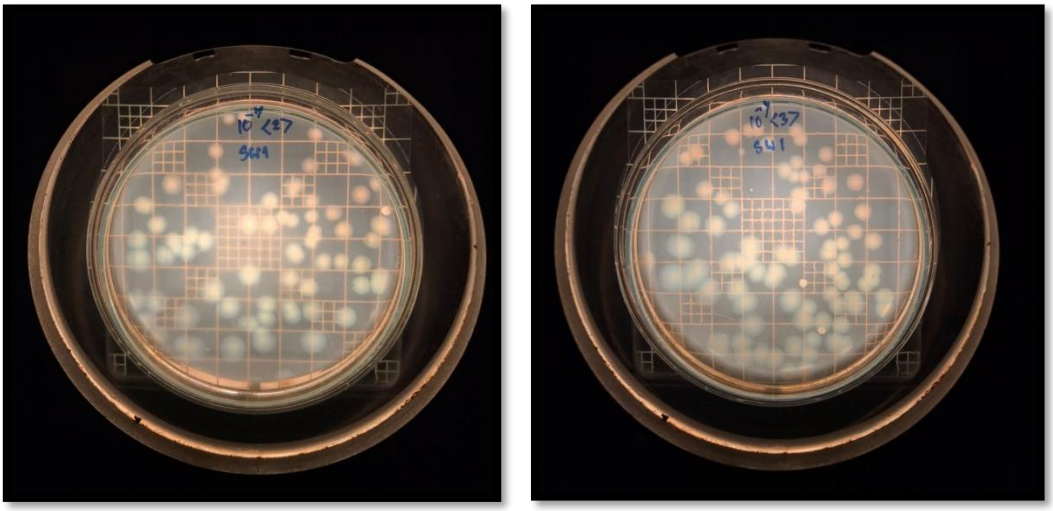

Figure 9 Colonies of SW1 concentration 10^{-7}

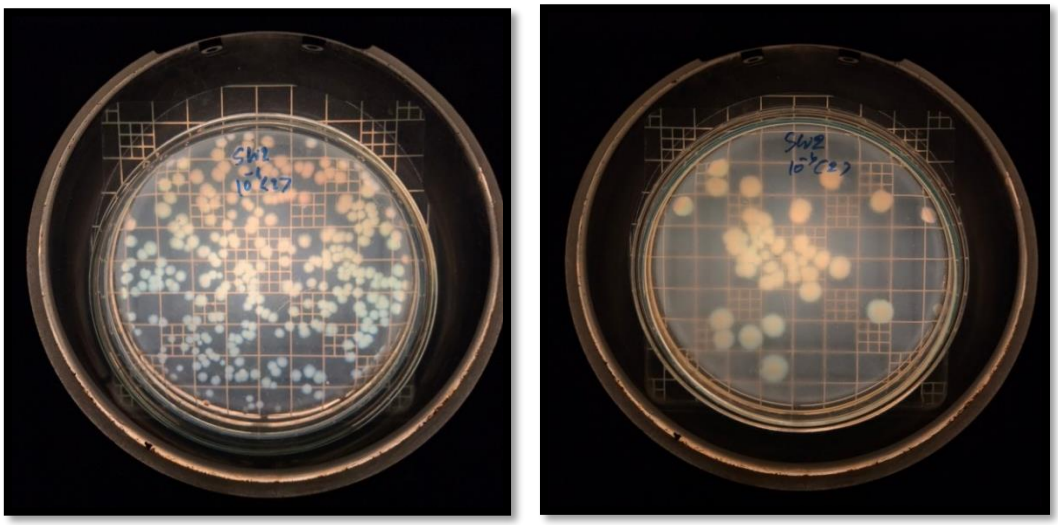

Figure 10 Colonies of SW2, on the left concentration 10^{-6}, on the right concentration 10^{-7}

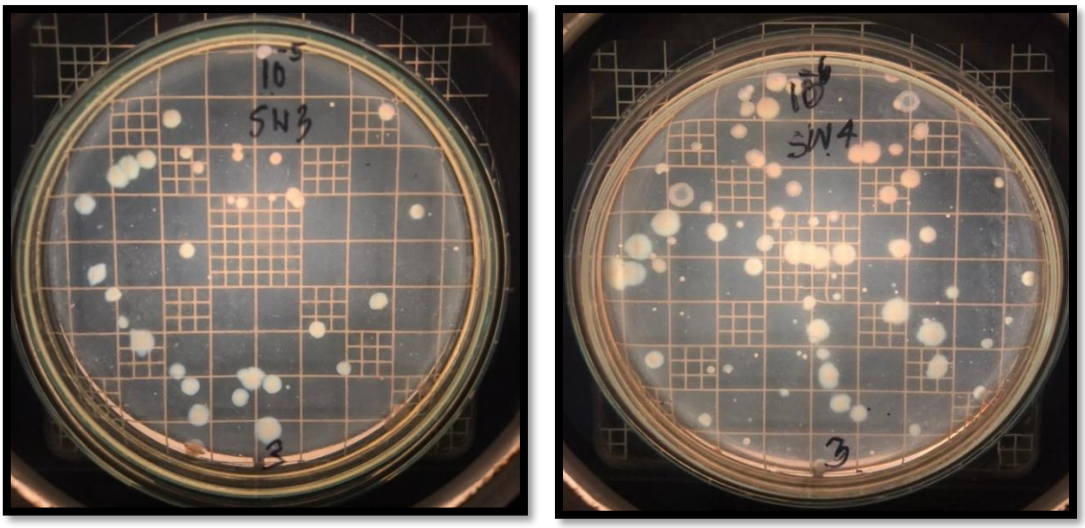

Figure 11 Colonies left of SW3 concentration 10^{-5}, colonies right of SW4 concentration 10^{-6}

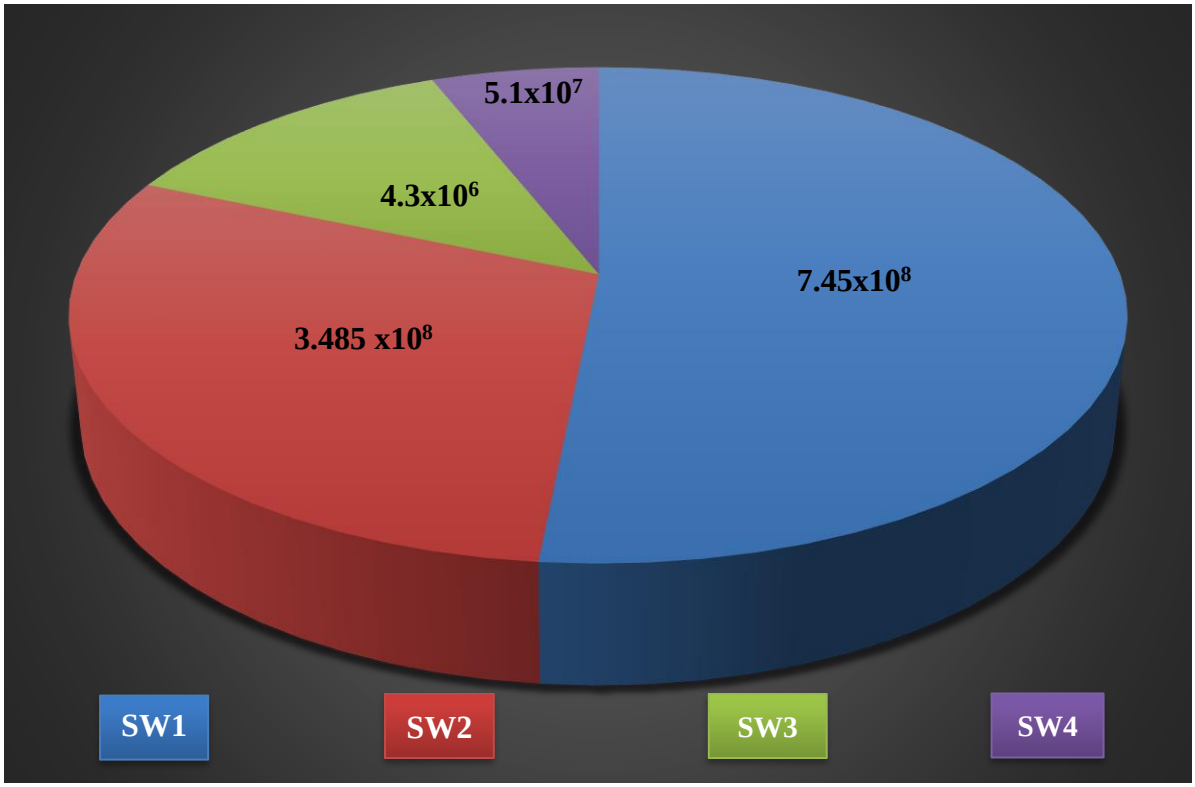

Figure 12 The comparison chart of the oil degradation capacity of the four bacteria.

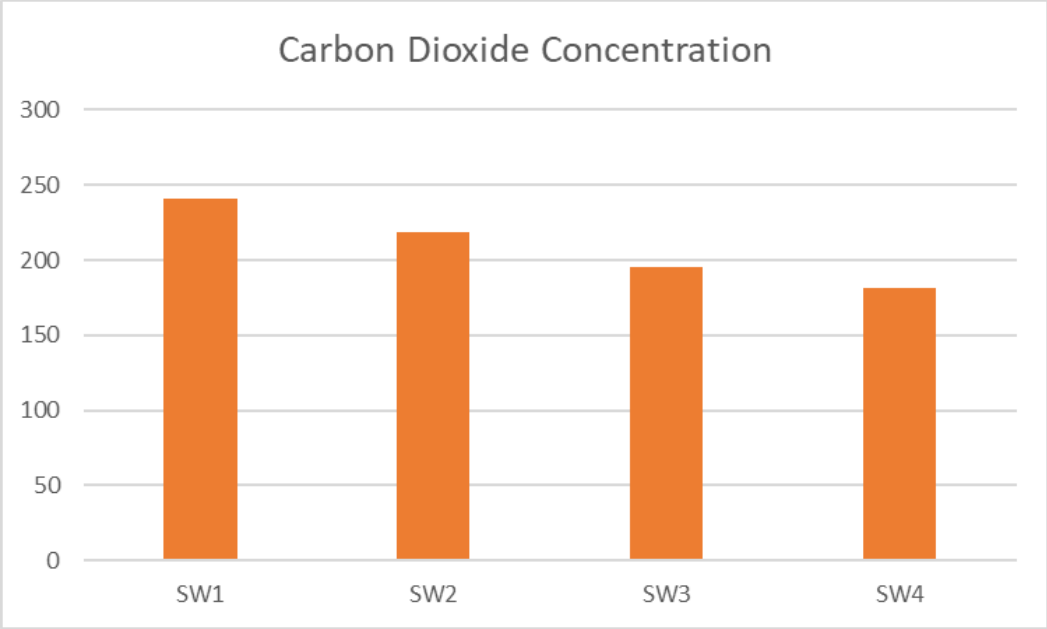

Figure 13 Carbon Dioxide Concentration

Analysis method	Sample volume	CO_2 Concentration			
		Sample 1	Sample 2	Sample 3	Sample 4
	mL	mmol L^{-1}			
GPFIA	1.00	122 ± 3	48 ± 1	178 ± 3	105 ± 1
	2.00	124 ± 5	50 ± 3	186 ± 5	106 ± 5
Titration	1.00	128 ± 4	47 ± 3	186 ± 4	102 ± 4
	2.00	125 ± 2	46 ± 3	184 ± 4	100 ± 2

Figure 14 Carbon Dioxide Concentration (Doran & Zander, 2012)

Figure 15 Salmonella on SS agar (source: www.microbiologyinfo.com.)

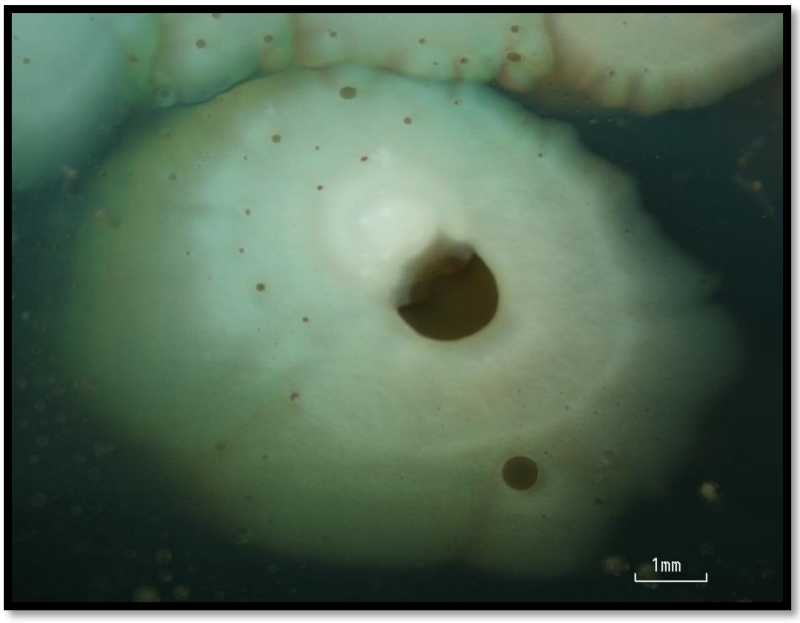

Figure 16 Bacterium colony is releasing enzyme degrade droplets of lubricant.

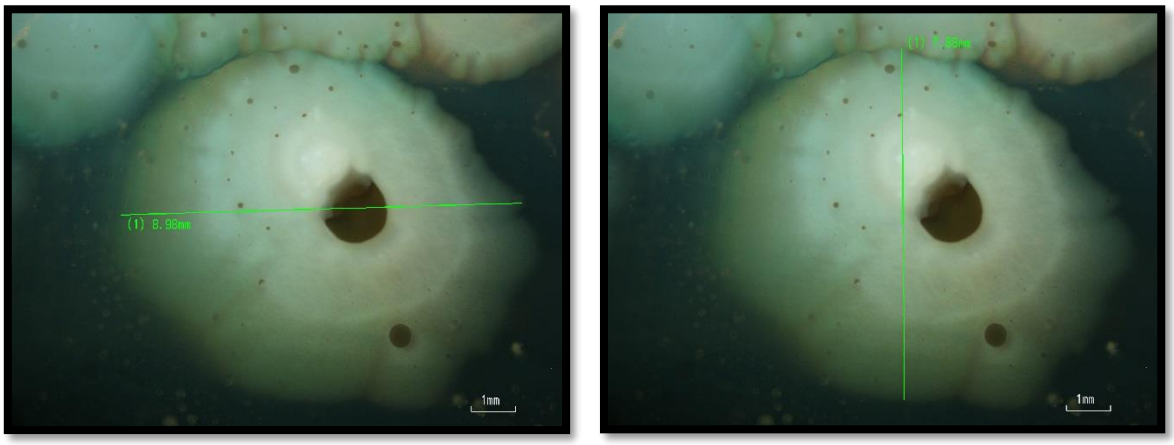

Figure 17 Diameter of colony

Assume using HCl to 9.63 ml.

$$\frac{44.01\ gm\ CO_2}{1\ mol\ CO_2} \times \frac{1\ mol\ CO_2}{2\ mol\ HCl} \times \frac{0.5\ mol\ HCl}{1000\ ml\ HCl} \times 9.63\ ml\ HCl = 0.106\ gm\ CO_2$$

Figure 18 Calculating the amount of carbon dioxide in grams.

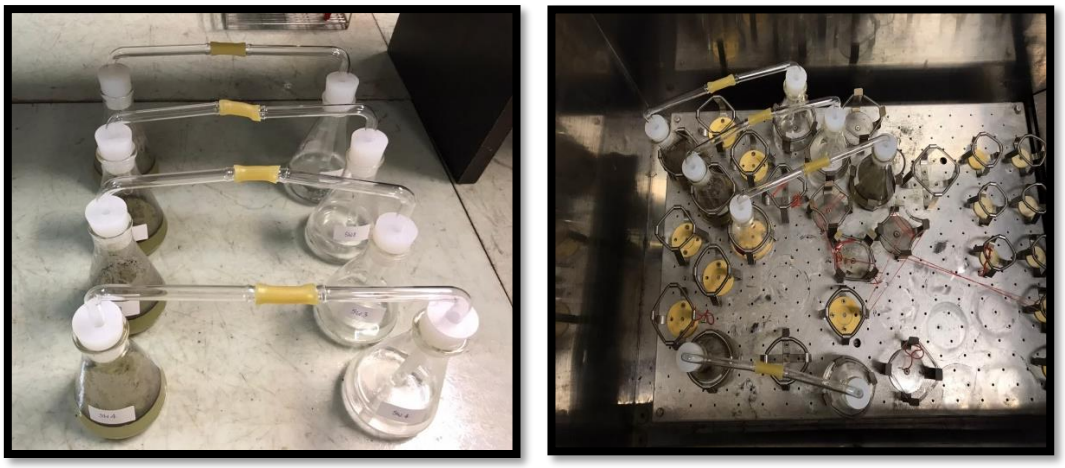

Figure 19 Equipment to detect carbon dioxide.

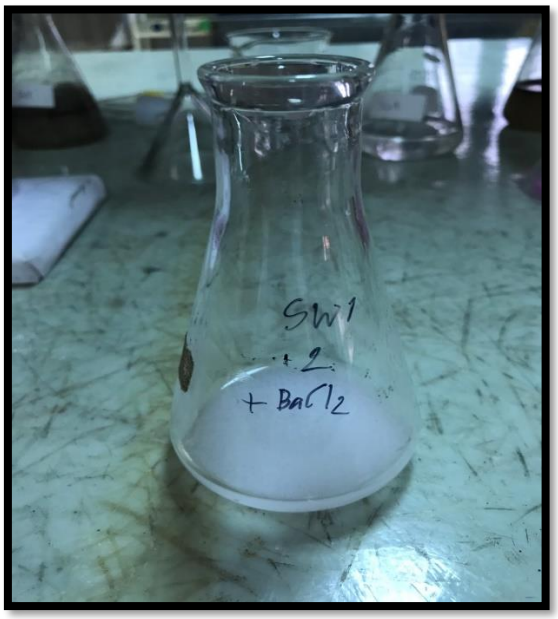

Figure 20 White precipitate occur from Ba_2Cl mixed with Na_2CO_3.

Figure 21 The color of the solution change to pink known as “End Point”.

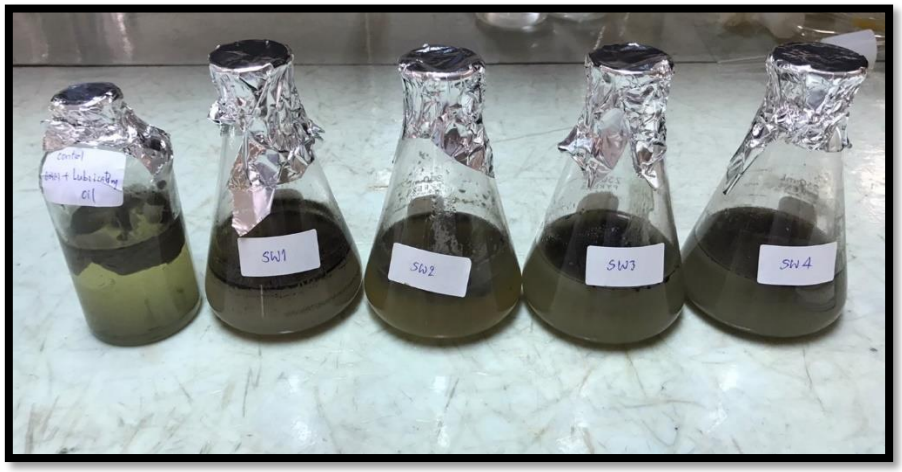

Figure 22 Showing color of culture media.

CHAPTER 5

Conclusions

Through this study, it was observed the ability of bacterial isolates from contaminated soil with spent lubricating oil to consume spent lubricant for a source of carbon through growth in media containing lubricating oil. SW1, SW2, SW3, and SW4 were isolated from contaminated soil. They were gram-negative. Detection of the consuming compounds using titration method showed SW1 was highest ability to degradation and SW4 was fewest ability to degradation. SW1 created amount of carbon dioxide 0.0106 gm/ml in 14 days follow by 0.0096 gm/ml of created amount of carbon dioxide by SW2, 0.0086 gm/ml of created amount of carbon dioxide by SW3 and 0.0080 gm/ml of creates amount of carbon dioxide by SW4. So, both objectives were achieved.

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Appendix

Bushnell-Hass medium (BHM)

$MgSo_4$ $7H_2O$	0.20 gm
K_2HPO_4	1.00 gm
$FeCl_3$	0.05 gm
NH_4NO_3	1.00 gm
$CaCl_2$	0.02 gm
Distilled Water	1000 ml

Trypticase soy agar (TSA)

TSB	30 gm
Agar	18 gm
Distilled Water	1000 ml

Alcohol-acetone (decolorizer)

Ethyl Alcohol	250 ml
Acetone	250 ml

Crystal Violet

Solution A

Crystal Violet (85% dry)	2.0 gm
Ethy Alcohol	20.0 ml

Solution B

Ammonium Oxalate	0.8 gm
Distilled Water	1000 ml

Mixed solution A and B 1:10

Gram's Iodine solution

Iodine (crystal)	1.0 gm
Potassium Iodine (KI)	2.0 gm
Distilled Water	300 ml

$BaCl_2$ 0.5 mol L^{-1}

$BaCl_2$ H_2O	2.828 gm
Distilled Water	25 ml

NaOH 1.0 mol L^{-1}

NaOH	40 gm
Distilled Water	1000 ml

HCl 0.5 mol L^{-1} (37%)

HCl	41 ml
Distilled Water	1000 ml

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