EFFECT OF CRUDE ALDEHYDE DEHYDROGENASE ON THE DEGRADATION OF CRUDE OIL BY A LOCAL ISOLATE, *ACINETOBACTER BAUMANNII* FETL C4

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LIST OF SYMBOLS

%	Percentage
°	Degree
^{o}C	Degree Celsius
μ	Micro
±	Plus minus
+	Plus
=	Equal
-	Minus

LIST OF ABBREVIATION

OPEC	Organization of the petroleum exportcCountries
PAH	Polyaromatic hydrocarbon
PDO	Polyaromatic hydrocarbon dioxygenase
C23O	Catechol 2, 3-dioxygenase
v/v	Weight per volume
pH	Power of hydrogen
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
API	American petroleum institute gravity
O_3	Ozone
PCB	Polychlorinated biphenyls
USD	United state dollar
U.S. EPA	United state environment protection agency
C32	Carbon 32
C20	Carbon 20
C5	Carbon 5
C12	Carbon 12
C10	Carbon 10
C-C	Carbon-Carbon bond
C-H	Carbon-Hydrogen bond
sMMO	Non-heme di-iron monooxygenases
pMMO	Membrane bound particulate copper-containing enzymes
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate

OCT plasmid	Octane plasmid
NAD^+	Nicotinamide adenine dinucleotide coenzyme
NADH	Reduced NAD^+
CPADH	*Candida parapsilosis* alcohol dehydrogenase
mL	mililiter
hr	Hour
min	Minute
rpm	Rotation per minute
w/v	Weight per volume
g/L	Gram per liter
Na_2HPO_4	Disodium hydrogen phosphate
L	Liter
lpm	Liter per minute
KH_2PO_4	Potassium dihydrogen phosphate
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate
$FeSO_4 \cdot 7H_2O$	Ferrous sulfate heptahydrate
nm	Nanometer
OD	Optical density
M	Molar
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
16S rRNA	16S ribosomal ribonucleic acid
HMDA	Hexamethyldisilazane
ERIC	Electronic rapid compendium

TEM	Transmission electron microscope
GC-FID	Gas chromatography flame ionization detector
ID	Internal diameter
ug/uL	Microgram per microliter
mg	Miligram
TPH	Total petroleum hydrocarbon
F1	Fraction 1
F2	Fraction 2
F3	Fraction 3
$(NH_4)_2SO_4$	Ammonium sulphate
NH_4NO_3	Ammonium nitrate
DTT	Dithiothreitiol
ALDH	Aldehyde dehydrogenase
sp.	Species

KESAN ENZIM KASAR ALDEHIDA DEHIDROGENASE TERHADAP PROSES DEGRADASI MINYAK MENTAH OLEH PENCILAN TEMPATAN, *ACINETOBACTER BAUMANNII* FETL C4

ABSTRAK

Pengesanan aktiviti enzim pengurai hidrokarbon daripada mikroorganisma yang berupaya menggunakan minyak mentah sebagai sumber karbon telah dikaji daripada pencilan mikroorganisma yang berpotensi daripada sampel air dan tanah dari kawasan yang telah dicemari di sekitar Pulau Pinang. Tujuh pencilan bakteria telah diperolehi dan dikategorikan sebagai bakteria Kumpulan I berdasarkan kepada ciri pencilan yang berupaya menunjukkan daya pertumbuhan yang baik apabila dikulturkan di dalam medium agar minyak mentah yang telah disediakan dengan menggunakan enam jenis minyak mentah sebagai sumber karbon yang dinamakan sebagai Tapis, Khefji Dubai, Bunga Kekwa, Angsi, Dulang and Penara. Medium agar minyak mentah ini disediakan tanpa penambahan surfaktan. Daripada tujuh pencilan yang diperolehi, pencilan bakteria pengurai minyak FETL C4 mempamerkan daya pertumbuhan yang lebih baik berbanding dengan pencilan yang lain di dalam medium minyak mentah. Hasil daripada ujian biokimia pencilan ini dikenalpasti sebagai *Acinetobacter sp.* Pengesahan selanjutnya telah dijalankan dengan menggunakan ujian analisa gen 16S rRNA dan Ujian Remel Rapid kit. Aktiviti enzim *aldehida dehidrogenase* (ALDH) telah dikesan semasa pengkulturan bakteria ini dalam media minyak mentah yang hanya mengandungi kandungan garam mineral dalam kuantiti yang minimum dan 1% (w/v) minyak Tapis sebagai unsur hidrokarbon. Pengoptimunan kadar pengeluaran enzim ini telah dilaksanakan dengan penentuan faktor fizikal dan kimia yang berupaya memberi rangsangan yang positif terhadap pertumbuhan dan penghasilan enzim oleh pencilan bakteria ini.

Proses pengoptimunan dilaksanakan dengan menggunakan sistem kelalang (250 mL) dan dilakukan dengan sistem fermenter (5 L). Sel-sel bakteria dituai apabila aktiviti enzim dan penguraian minyak mentah mencapai kadar maksimum. Keadaan optimum bagi activiti enzim mencapai kadar maksimum telah ditentukan apabila 0.2 g/L $(NH_4)_2SO_4$, 0.4 g/L KH_2PO_4, 0.8 g/L Na_2HPO_4, 1.0 g/L minyak mentah Tapis, 0.2 g/L Tween 80 sebagai surfaktan, suhu pengkulturan pada 37°C, pH awal medium pada 8.0, dengan penambahan 5% inokulum (1 x 10^6 sel/ml) dan digoncangkan pada 600 rpm. Kadar pengudaraan disediakan pada kadar 2 L per minit untuk meningkatkan kadar pertumbuhan kultur bakteria ini. Kadar penghasilan biomass dan kadar penguraian minyak mentah oleh kultur bacteria masing-masing pula adalah pada 2.0 g/L and 70%. Analisis zimogram pada enzim aldehida dehidrogenase ke atas substrat aldehida yang berlainan hanya menunjukkan kemunculan satu jalur pada gel zimogram dan seterusnya menunjukkan jalur ini terlibat dalam metabolisme pelbagai substrat aldehida. Analisa kuantitatif pada enzim in menunjukkan kecenderungan enzim yang tinggi pada substrat yang mengandungi 12 karbon.

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ABSTRACT

Detection of hydrocarbon-degrading enzymes activities in microorganisms capable of utilizing crude oil as a carbon source were done using potential oil-degraders isolated from soil and water samples collected from water and oil-contaminated areas in Penang. Seven potential isolates categorized as Group I bacteria were selected based on the criteria that they displayed good growth on solid crude oil medium using 6 different types of crude oil which were named as Tapis, Khefji Dubai, Bunga Kekwa, Angsi, Dulang, and Penara. These crude oil media were prepared without the addition of surfactant. Out of seven isolates, oil-degrading bacterium designated as FETL C4 displayed good growth on different types of crude oil media prepared. Biochemical test results identified this isolate as *Acinetobacter* sp. Further confirmation was done using 16S rRNA gene analysis and Remel Rapid Identification Kit. Aldehyde dehydrogenase (ALDH) activity was detected during the cultivation of this isolate when grown in minimal salt medium containing 1% (w/v) Tapis crude as a carbon source. Optimizations on the production of this enzyme were done by manipulating physical and chemical parameters of the growth and enzyme production. The optimization process were carried out in a flask system (250 mL) and then further scaled up via fermenter system (5 L). Cells were harvested from the growth medium when maximum points were reached for both enzyme activity and degradation of the crude oil. The optimal conditions when aldehyde dehydrogenase activity detected at the highest rate were obtained at 0.2 g/L

$(NH_4)_2SO_4$, 0.4 g/L KH_2PO_4, 0.8 g/L Na_2HPO_4, 1.0 g/L Tapis crude, 0.2 g/L Tween 80 as surfactant, cultivation temperature at 37°C, initial medium pH 8.0, with addition of 5% inoculum (1 x 10^6 cells/mL) and agitated at 600 rpm. Aeration rate was set at 2L per minute to enhance the growth of the isolate. Biomass production and degradation rate of the crude oil were 2.0 g/L and 70%, respectively. Zymogram analysis of aldehyde dehydrogenase on different aldehyde substrates revealed that only one activity band that involved in the metabolism of various aldehyde substrates. Quantitative analysis of this enzyme on different aldehyde substrates showed a higher preference towards aldehyde with carbon 12.

CHAPTER ONE

INTRODUCTION

1.1 Current status of oil spills and the solutions

Oil pollution occurs either accidentally or intentionally from ships transportation, natural slicks or pollution from land during drilling process or repair operations. Source of these problems arise due to reckless attitude of human being. Oil leak from ships and tankers during transportation is the major contribution of oil spills happening in the ocean. Nevertheless, terrestrial pollution is also important as it will also cause devastating effect to the offshore environment. It has been estimated that the world oil consumption will be around 91 million barrels per day in 2010 and will reach to 103 millions barrels a day in 2020 as compared to 76 millions barrels per day during the year of 2001 (OPEC, 2008). Large scale production and usage of petroleum globally has also caused extensive environmental contamination. There are three possible sources of petroleum pollution in Malaysia, oil tanker sources in the straits of Malacca, domestic oil production and rapid industrialization. The major oil spills in Malaysia are due to oil tanker accidents. On May 28, 2001, an oil tanker with 67 tones of fuel sunk after it was crashed from behind by a super tanker near Malacca straits. The fuel spread to about one nautical mile from the collision spot. About two weeks later, another oil spillage occurred in Southern Johor. Oil spillages not only cause an immediate effect to the environment and biological life but also a long term devastating effect to the ecological system by destroying or interrupting the food chain of the wild life system. Indirectly it will also result in socio-economic impact yet to be determined in different sectors.

The current practice used in cleaning up oil spillage is the implementation of biodegradation and bioremediation concepts. Bioremediation is a process that uses microorganisms to transform harmful substances into non-toxic carbon dioxide, water and fatty acids. With the advances in technology, many attempts have been made to accelerate the process of bioremediation in order to eliminate toxic materials within in a short period of time. This technique causes less or no harmful effect to the environment and ecological system as compared to other conventional techniques used in previous years as the microbes. Attempts of using physical or chemical containment such as skimmers or boomers will damage the specific environment as located in Malaysia, a country rich with coastal beaches and mangrove flats. Moreover, using booms and skimmers may take many hours to achieve as to organize responsible committee for the action. The only effective treatment was bioremediation using microorganisms and enzymes. Since the vast majority of crude oils consist of hydrocarbon that are degradable, bioremediation can be environmentally responsible, less invasive, cost effective way of restoring sites contaminated with oils and fuels (Baker and Herson, 1994; Seah *et al*., 2001). Bioremediation have an edge over the physical-chemical treatment regime as they offer *in-situ* biodegradation of oil fractions by microorganisms (Ferrari *et al*., 1996). The practice of using bioremediation in cleaning up oil spills have been shown by one of the company in United States of America. Alabaster Corporation have been producing microbial blend product for use in bioremediation process. This product contains the desirable microbes, enzymes and nutrients required to accelerate the process of biodegradation (Alabaster Bioremediation Corporation, 2005). Nutrients were required to stimulate the growth of microbes whereas the enzymes facilitate the biochemical processes and help the microbes to obtain the desirable carbon source.

Isolation of the potential oil degrading microorganism from contaminating sites in Malaysia can be used to study for the production of enzymes involved in the process of breaking hydrocarbon compounds. The protein catalysts can increase the chemical reaction without altered itself. Hydrocarbon degrading enzymes are secreted or produced by microorganisms during fermentation process and posses the ability to break down bonds within organic compounds. Various studies have been carried out on enzymes capable of degrading different types of hydrocarbon compounds. Some examples of these hydrocarbon compounds are the short-chain and long-chain aliphatic hydrocarbons while aromatic hydrocarbons are benzene, toluene, ethyl benzene, phenanthrene and naphthalene and also chlorinated derivatives (Singer *et al*., 1985; Andreoni *et al*., 2004). The initial attack on hydrocarbon compound is an oxidative process catalyzed by oxygenases and peroxidases. Polyaromatic hydrocarbon (PAH) dioxygenase (PDO) and catechol 2, 3-dioxygenase (C23O) were identified as key enzymes in polyaromatic hydrocarbon (PAH) biodegradation (Resnick *et al*., 1996; Meyer *et al*., 1999). Whereas in alkane hydrocarbon dissimilation metabolic pathway, alkane hydroxylase, alcohol dehydrogenase, aldehyde dehydrogenase, acyl-CoA synthetase are essential for this pathway (Singer *et al*., 1985; Kok *et al*., 1989; Van Beilen *et al*., 1994). On the entire enzyme involved, aldehyde dehydrogenase is recognized as an important enzyme for oxidation of hydrocarbon and other various compounds (Okibe *et al*., 1999). Therefore, in the present study, the production of aldehyde dehydrogenase was performed.

Enzyme production can be very cost effective as they minimize waste disposal and heating process needed in the reactivating microorganisms (Gianfeda and Rao,

2004). Unlike many microbes, enzymes remain effective in a wide range of pH and temperature. They are able to act in a large range of environmental conditions and remain active even if these conditions quickly change. Action for cleaning up oil spills have to be carried quickly to avoid spreading of the oil to the offshore and formation of tar balls. Designing and production of enzyme powder from potential microorganism can be time saving as it helps to accelerate the biodegradation process. Moreover, enzymes themselves are biodegradable proteins, as they can be degraded in the environment after they are no longer needed (Ahuja *et al*., 2004).

1.2 Research objectives

The research objectives of the study are:

i. To isolate, screen and identify the petroleum hydrocarbon-degrading microorganism
ii. To optimize the aldehyde dehydrogenase production using shake flask and fermenter systems
iii. To characterize crude aldehyde dehydrogenase obtained

1.3 Research scopes

Microorganisms of interest were isolated from oil contaminated sites in Seberang Prai and Penang Island. These microorganisms were screened for their capabilities in utilizing various crude oil by culturing in six different types of crude oil provided by PETRONAS Research Sdn. Bhd. The designated crude oils were sweet crude Tapis, light crude Angsi, heavy crude Bunga Kekwa, medium crude Dulang, Penara medium crude and Khefji Dubai Sour crude. The pre-selection of the microorganisms were qualitatively determined based on their growth in crude oil

medium prepared in solid agar form. Post-selection were quantitative determined based on their growth and degradation rate in 1% oil medium broth (v/v) prepared with minimal basal salts. The selected microorganisms were tested using Tapis crude for their capabilities in enzyme production study. Time course profile of the microorganism before and after the optimization process were evaluated based on their growth, degradation rate and enzyme activity. The optimization process was performed using shake flask system in order to determine the conditions for optimal enzyme production. The parameters optimized were; temperature, pH, inoculum size, agitation speed, crude oil concentration, types of nitrogen source and its concentration, types of phosphorous source and its concentration and types of surfactant and its concentration. The cultivation via fermentation system was performed to evaluate the effect of agitation, aeration and the effect of crude oil concentration on enzyme production, growth and degradation of Tapis crude. Air was continuously supply to the fermenter since the air in a flask system was limited. Zymograph analysis of the targeted enzyme was done on different aldehyde substrates to study the substrate specificity of the enzyme. Molecular size determination of the enzyme was then determined using Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis (SDS-PAGE) method by excising the targeted enzyme from native-gel electrophoresis. The crude enzyme was quantitatively characterized in terms of their substrate specificity, pH and temperature activity and stability.

CHAPTER TWO

LITERATURE REVIEW

2.1 CRUDE OIL

2.1.1 Type of oils and their properties

Oil is a general terms that describe a wide variety of natural substances of plants, animals, or mineral origin. Different types of oil are made of different major compounds and many different minor substances. As their compositions are different, therefore each type of oil has its certain unique characteristics that bring about how they behave when spills, effects on living organism in the environment and the efficiency for cleanup process. Crude oil are mixtures of hydrocarbon compounds ranging from smaller, volatile compounds to very large, non-volatile compounds. Hydrocarbon is an organic compounds consisting entirely of hydrogen and carbon (Fingas, 2001). In chemical terminology, aromatic hydrocarbons, alkanes, alkenes and alkyne-based compounds are referred as pure hydrocarbons which composed entirely of carbon or hydrogen whereas other hydrocarbons with bonded compounds such as sulfur or nitrogen were impure hydrocarbons. The majority of hydrocarbons can be found naturally in crude oil.

Crude oil is formed from several naturally derived organic materials under the influence of high pressure and temperature over geological time scales (Wise *et al*., 2000). The difference in chemical compositions and properties in crude oil was due to the variable nature of these factors. Owing to the fact that crude oil is of different composition, their biodegradability also varies. Sugiura *et al.* (1997) concluded that different biodegradability in different types of crude oil was due to differences in

their compositions. The experiment was carried out in four different types of crude oil mainly, Arabian Light crude, Maya, Shengli and Dubai crude by *Acinetobacter* sp T4 and microbial consortium called SM8.

The proportion of hydrocarbons in the mixture is highly variable and ranges from as much as 97% by weight in the lighter oils to as little as 50% in the heavier oils and bitumen. The hydrocarbons compounds found in crude oil are mostly alkane, cycloalkanes and various aromatic hydrocarbons while other organic compounds are nitrogen, oxygen sulfur and trace elements such as iron, nickel, copper and vanadium (Speight, 1999) which hydrocarbon made up of 83% to 87% of the entire compounds. There are four different types of hydrocarbon which can be found in crude oil namely paraffins, aromatics, naphthenes and asphaltic. Each of these compounds varies from oil to oil (Hyne, 2001).

Different types of crude oil are well represented with different properties. The properties of oil discussed are the viscosity, density, specific gravity, solubility, flash point, distillation fractions, interfacial tension and vapour pressure. These parameters are important to distinguish between different types of oils (Fingas, 2001). For example, heavy crude is one type of crude oil which can be differing from other crude as it does not flow easily. It is referred as heavy because its density or specific gravity is higher than light crude oil or medium crude oil. Heavy crude oil has been defined as any crude with API gravity less than 20° (Dusseault, 2001) which mean that their specific gravity is greater than 0.933. Oils with high densities have low API values.

2.1.2 Behavior of crude oil in environment

Crude oil behavior in the environment is referred to as number of transformation processes when the oil is spilled whether on land or water. Ultimately, there are two types of transformation processes; the first is the weathering processes where the physical and chemical properties of the oil change after the spill. The second is related to the movement of oil in the environment. Both are important in determine how the oil should be clean up and its effect on the environment (Basic of oil spill cleanup).

Fingas (2001) reported that weathering process undergoes different rates but begin immediately after the spill. The process rates are not consistent throughout the duration of an oil spill and usually highest immediately after the spill. The processes included in weathering are evaporation, emulsification, natural dispersion, dissolution, photooxidation, sedimentation, adhesion to materials, interaction with mineral fines, biodegradation and the formation of tar balls. Dispersion is always associated with the wave formation in aquatic spill, breaking a slick into droplets which are then distributed throughout the water column. These droplets can also form a secondary slick or thin film on the surface of the water. Evaporation occurs when lighter or more volatile substances within the oil mixture become vapours leaving behind the heavier components of the oil. Oxidation occurs when oil droplets contacts with the oxygen and hydrocarbon in the oil components which turn into water soluble compounds. Mostly oxidation process affects oil slicks around their edges. Emulsification is process which forms emulsion of small droplets of oil and water. This process is very important as they greatly hamper weathering and cleanup process. Oil and water emulsions can cause oil to sink and disappear from

the surface, giving a false indication that the treat to the environment has ended (EPA, 2008). Biodegradation is the process when microorganism feed on the oil hydrocarbons for energy and growth tends to work best in warm water environment.

Weathering and movement processes can be overlap, with weathering strongly influencing how oil is moved in the environment and vice versa. These processes depend very much on the type of oil spilled and the weather conditions during and after the spill. For example, this process can occur differently in standing water environment to marine environment. Standing water environment can be more severe because water movement is minimized in this habitat and can remain in the environment for a long period of time. Oil can also interact with the sediment at the bottom of the freshwater bodies, affecting organism that live in or feed off the sediments. Figure 2.1 illustrates the behavior of oil spilled in the environment. The natural weathering conditions can help to reduce the severity of an oil spill and accelerate the recovery of an affected area. Some natural actions include *weathering, evaporation, oxidation, biodegradation*, and *emulsification* as illustrated in the figure.

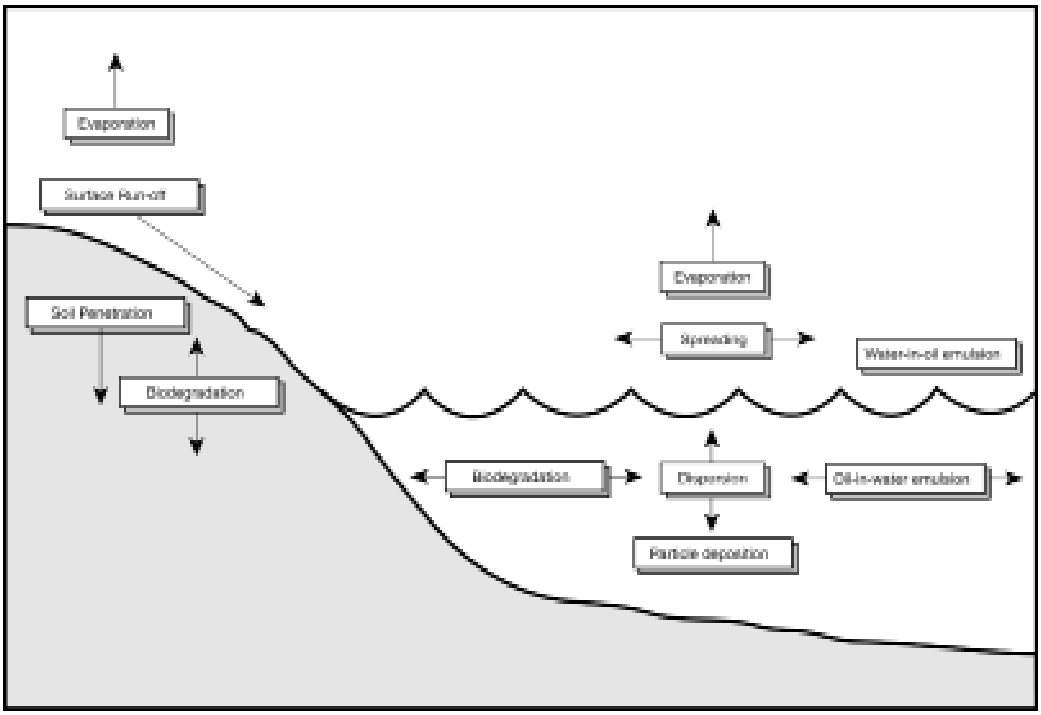

Figure 2.1: Behavior of oil in spilled environment (EPA, 2008)

2.2 EFFECT OF OIL SPILLS TO ENVIRONMENT

The toxicity of some of the substances found in the petroleum compounds can bring about many adverse effects on the environment. Toxic effects can be classified as chronic or acute, which refers to the rate of effect of toxin on an organism. Acute means effects that occur within a short period of time in relation to the lifespan of the organism and could bring death. Chronic means occurring during a relatively long period in relation to the lifespan of the organism, this effect are usually related to changes in metabolism, growth, reproduction or the ability to survive (Fingas, 2001). Some toxic substances in an oil spill may evaporate quickly, therefore plants and animals that expose to these substances can be reduced with time. Although some organism may be seriously injured or kill very soon after contact with the oil spill, non-lethal toxic effects or chronic effects can be more subtle and last longer.

There exist complex interrelations between plant, animal species and their physical environment. Harm to the physical environment will often lead to harm for one or more species in a food chain, which may lead to damage for other species related in the food chain. This can be generally explained as bioaccumulation. The accumulation of toxin in the flesh are rarely occurs since the components of oil are generally metabolized by receiving organism (Fingas, 2001). In an aquatic habitat, fish have the ability to swim away from the polluted area, reducing the likelihood they will be harmed by even major spill, but for the organism that live near the shoreline such as the turtles, risk contamination by oil that washes onto the beaches or by consuming oil-contaminated prey. Moreover, oil may harm sea grass or coral reefs which are used for food, shelter and nesting sites by many different species. The most visible biota that was affected by the oil spills is the bird species. Birds

are easily contaminated by oils onto their feathers when come into contact with the slicks on water or shorelines. Bird species can loss their body heat rapidly as their insulation and buoyancy properties decreased which will lead to death. Contaminated birds may transfer oil to their eggs or young too. It has been found that only a few drops of fresh oil can kill the young in egg.

2.3 CURRENT PRACTICE IN OIL SPILL CLEANING

2.3.1 Physical and Chemical method

Physical and chemical methods for oil spill cleanup have been extensively used for a long time with varying degree of success. These methods include booming, skimming, sorbent agents, dispersant, sinking agents and also in-situ burning. Booms are employed to prevent oil from spreading on the water's surface and surround the spill close to the source. They are used to prevent the oil from entering the harbor, docks or any sensitive areas and to divert the oil spill to an area where operation can be made (NOAA-a, 2004). Different from booming, skimmers are designed to recover oil from the surface of the water. With this technique, spilled oil removed from the environment can be collected and recycled (USCG, 2004). However, both of these techniques bring about some difficulties when dealing with spill at open sea area. Both techniques can be considerably limited by several environmental factors such as winds, waves and currents and is also complex, expensive and labour extensive (Fang and Johnson, 2001; Mulin and Champ, 2003). Sorbents are used together with other mechanical methods such as skimmers to recover and clean up oil spills by the adsorption (or/and) absorption of the oil. But yet this method of cleaning oil spillage do have some disadvantages such as poor floating characteristics, relatively low sorption capacity and low hydrophobic (Choi

and Cloud, 1992; Adebajo *et al.*, 2003). Especially crude oil, consists of varying mixtures of hydrocarbons, sorbents can strip only the lighter fractions, leaving the heavier components unstripped (Smith, 1983). Moreover, disposal and recovery of the absorbing materials is also a factor to consider (Mullin and Champ, 2003).

Chemical methods can be categorized into several major groups (Ventikos *et al.*, 2004): dispersing agents, sinking agents (sand, brick-dust or even cement) and others (gelling agents, emulsion breakers). Dispersants are used more commonly as comparing to others chemical methods. However, the dispersion of an oil spill is not actually oil removal since it only breaks the oil up into finer particles, resulting in an increase in oil concentration in water around the oil spill considerably (IPIECA, 2001; Christodoulou, 2002) that may have a negative impact on the flora and fauna. Sinking agents such as sand, brick-dust, fly ash, china clay, volcanic ash, coal dust, stucco, slaked lime, spent tannery lime, crush stone and cement (Dewling, 1980; Smith, 1983) also encountered some problems. Many sinking materials do not keep the oil permanently immobile and release of the oil, causing re-pollution after some time. Sinking agents are also not applicable in enclosed water or shallow water (less than 100m deep), in which the volume of water is not sufficient to prevent the oil particles from reforming an oil slick again. In addition, oil that sinks to the bottom contaminates benthic life and degrades more slowly than when they are floated, dispersed, or dissolved in water (NRC, 1989). Oil spills are also treated by burning-off (Mullin and Champ, 2003). Under favorable conditions, this method is efficient, fast and a relatively simple way for oil spill removal. However, burning oil is not an environmentally friendly method as it causes air pollution and leaves residual un-burnt particles (Smith, 1983; Christodoulou, 2002).

2.3.2 Biological method

Bioremediation of oil spills is the process of using living organisms to degrade pollutants and recover environmental quality (Atlas and Cerniglia, 1995). In recent years, biological techniques have been used extensively. This method has an advantage of generating no further negative environmental impact (Wood *et al.,* 1997), uses natural processes, transforms contaminants instead of simply moving them from one media to another and is affordable (Senn, 1999). Bioremediation techniques have become a major mechanism for removing oil residues on the affected shorelines. Among the different techniques to enhance natural biodegradation by indigenous microorganisms, seeding of new bacteria and fertilizing the indigenous populations have attracted the most interest. The application of nutrients as nitrogen and phosphorus in the form of fertilizers have shown to be most effective in accelerating the biodegradation process and at the same time to be environmentally safe. Much research regarding biological treatment of oil spills has been done, including microbial surfactants (Harvey *et al.*, 1990), bacterial consortia (Chatre *et al.*, 1996) and marine microbial mats (Cohen, 2002). Bioremediation of oil spills and petroleum contaminants have also been investigated by Atlas and Cerniglia (1995), Prince (1993), Head and Swannell (1999); Tsutsumi *et al.* (2000). In particular, biological treatment of oil spills in cold environments has also been studied (Margesin and Schinner, 1999).

It is particularly important to address oil polluted waters as soon as possible as the contamination can have the potential to damage fishery resources and affect the health of those animals and humans that consume contaminated fish (Krahn and Stein, 1998). Besides the varying rates of biodegradation, researchers have

consistently documented a lag time after oil is spilled before indigenous microbes begin to break down the oil molecules. This lag time is related to the initial toxicity of the volatile fractions of the oil, which evaporate in the first few days of a spill. Microbial populations must begin to use oil and expand their population before measurable degradation takes place, a period usually lasting several days. This fact becomes very important when considering the appropriateness of bioremediation as a quick or first response technique (Hoff, 1993).

2.3.3 Combination of microbial and non-biological method

There are few reasons bioremediation often fails to effectively or extensively destroy individual organic pollutants or one or many in a mixture of compounds in a waste steam. Possibilities are maybe: (a) the single compound of concern may be intrinsically non-biodegradable; (b) the waste stream contains a number of organic substances, some of which are not readily biodegradable; (c) inhibition occurs due to sufficiently high concentrations of individual or the mixture of compounds that is degradable; (d) accumulations of organic product that are not readily metabolized by the organism present (Alexander, 1999). To overcome the concern, non-biological step usually comes first before the bioremediation take place (Scott and Ollis, 1995).

The non-biological method usually involved treatment with O_3, hydrogen peroxide, ultraviolet light, sunlight or Fenton's reagent (Alexander, 1999). The reasons underlying the used of non-biological method are stated above. If the compounds in the contaminated area are intrinsically non-biodegradable, the abiotic or non-biological method could help in converting abiotically the compound to products that readily metabolized. Abiotic techniques could also assist in reducing the

toxicity of the compounds to an extent that bioremediation is feasible. In addition, should some chemical treatment or sorption be needed for this multicomponent stream, a greater degree of biodegradation would reduce the cost of that subsequent treatment or increase the life of the sorbent. Ozone (O_3) pretreatment had been applied in the initial phase of treatment of slowly biodegraded or non-biodegradable chlorinated and nitro-containing benzenes and phenols as reported by Stockinger *et al.* (1995) and Adams *et al.* (1997) whereas ultraviolet irradiation was used in destroying PCB congeners in solution (Shimura *et al*., 1996).

In some cases, non-biological methods follow after the biodegradation procedure. The initial first step is relatively inexpensive procedure to reduce the amount of material that has to be destroyed in the second step (Alexander, 1999). This had been evaluated in a laboratory study involving microcosms containing samples of soils and suggested that the remaining of PAH after bioremediation were eliminated by Fenton's reagent (Stokley *et al.* 1997)

2.4 PRINCIPLES OF BIOREMEDIATION

Bioremediation is the used of microorganism to reduce the concentration and toxicity of various chemical pollutants, such as petroleum hydrocarbon, polycyclic aromatic hydrocarbons, polychlorinated hydrocarbon, phenyls, phthalate esters, nitroaromatic compounds, industrial solvents, pesticides and solvents (Glazer and Nikaido,1995; Eweis *et al*., 1998; Dua *et al.,* 2002). With the advanced in biotechnology, bioremediation has become the most rapidly developing fields of environment restoration. Basically, the selection of the most appropriate strategy to treat a specific contaminated site can be guided by three basic principles:

biochemistry, bioavailability and bioactivity. Biochemistry refer to the amenability of the pollutant to biological transformation to less toxic products, bioavailability is the accessibility of the contaminant to microorganism and bioactivity can be defined as the opportunity for optimization of biological activity (Dua *et al*., 2002). Bioremediation has been successfully used to treat petroleum-contaminated soils for the last 30 years (Ryan *et al*., 1991).

2.4.1 Types of bioremediation

Bioremediation is a new field of technology. The success of bioremediation is often measured by the percent reduction in contaminant concentration in soil or groundwater. The physical conditions involved in bioremediation process can be classified as in situ, solid phase or bioreactors. Treatments process used in bioremediation of contaminated soils, gases and water differ considerably. There are few types of bioremediation process in current used and practice. In ground water bioremediation, methods used can be categorized into pump and treat or in situ treatment. Pump and treats system pumped the groundwater to the surface and treated and follow either used directly or returned to the aquifer. This system always involved aeration, addition of nutrients and sometime the seeding of microorganism capable of degrading the contaminants in the groundwater. In situ treatment is a process that occurs in the subsurface. This kind of activity is stimulated by the introduction of oxygen and nutrients but often oxygen are found to be the most limited factor in subsurface biodegradation and can be introduced by ways such as air sparging or through the use of hydrogen peroxide. Bioremediation of soils can be carried out in situ or the material can be excavated and treated on site or at a separated treatment facility. In situ method includes soil venting and bioventing

where semivolatile and non-volatile contaminants are involved. In situ soil bioremediation requires transporting of oxygen and nutrients. Ex situ process in soil remediation includes land treatment, compositing and slurry phase bioreactors. Land farming involves aeration and mixing of contaminated soil by tiling, addition nutrients and control of moisture content by periodic addition of water. In composting, the contaminated material is mixed with organic bulking agents such as manure. Bioreactors are slurry phase operations in which the contaminated soils can be placed in a containment vessel and enough water is added to allow continues mixing.

2.4.2 Factors influencing bioremediation

The rate of microbial degradation of crude oil or oil waste depends on a variety of factors, basically they can be divided into three major factors, environmental, physical and chemical factors. Environmental factors are those necessary to provide optimum growth conditions to the microorganism to accelerate the bioremediation process. Microorganism's growths are affected by temperature, pH, nutrient availability, oxygen and moisture content. Different microorganism tends to have different optimum growth temperature and pH. They also required additional nitrogen and phosphorus that are limited in remediation sites for growth. The most important physical factors in bioremediation are the availability of contaminants to microorganism, presence of water and supply of a suitable electron acceptor. In line close relations among physical and environmental factors are the oxygen and moisture content. Water is required as a physical factor affecting the bioremediation process because microorganism obtain organic carbon, inorganic nutrient, and electron acceptor required for microbial growth from the liquid phase whereas

oxygen is another physical factor that is most widely used as a terminal electron acceptor for microbial respiration. These factors involving microorganism growth will be discuss further in section 2.5.2. The most important chemical factors in bioremediation are the molecular structure of the contaminant, its chemical and physical properties and its biodegradability. Biodegradability is related to factors such as solubility, degree of branching, degree of saturation, and the nature and extent of substitution. A generalized sequence of petroleum components in order of decreasing biodegradability is represented as follows: n-alkanes> branched-chain alkanes > branched-alkenes > low-molecular weight n-alkyl aromatics > monoaromatics > cyclic alkanes > polynuclear aromatics > asphaltenes (Huesemann, 1995).

2.4.3 Advantages of bioremediation

Bioremediation technique offers several advantages over the physical and chemical techniques used in cleaning contaminated area. Approaches such as air stripping and incineration may not be as cost effective comparing to bioremediation technique when one or more compounds contaminate a large area in low but significant concentrations (Dua *et al*., 2002). As had reported by Gabriel (1992), cleanup process using bioremediation only costs 100 USD to 250 USD per cubic meter while more conventional technologies such as landfilling may cost in the range of 250 USD to 1000USD per cubic meter. Biotechnology processes for the bioremediation of chemical pollutants offer possibilities of in-situ treatment and are mostly based on the natural activities of microorganism (Eweis *et al*., 1998; Dua *et al*., 2002). When successfully operated, this processes may achieved a complete destruction of organic pollutants as this technique is aimed at biodegrading and detoxifying hazardous

contaminant, whereas other technologies such as venting, adsorption onto activated carbon, solidification/stabilization, soil washing and disposal into landfills simply transfer contaminants to a different medium or location (Eweis *et al*., 1998). Consideration on bioremediation is the difficulty in predicting the performance of the microorganism in an environment contaminated with certain hazardous compound (Iwamoto and Nasu, 2001; Dua *et al.,* 2002). Microorganism are very sensitive to temperature, pH, contaminant toxicity, contaminant concentrations, moisture content, nutrient concentrations and oxygen concentrations as had discuss earlier in section 2.4.2. Many approaches have been improved in order to increase the efficiency of biodegradability in bioremediation technique; some are of biochemical and molecular studies (Bollag and Bollag, 1992; Johri *et al*., 1996). Furthermore, biotransformation of organic pollutants has been studied in detail in order to understand the microbial potential in bioremediation (Johan *et al*., 2001; Mishra *et al*., 2001; Watanabe, 2001)

2.5 PETROLEUM DEGRADING MICROBES AND THEIR RELATIONS TO BIODEGRADATION

2.5.1 Oil utilizing microorganism

Microorganism plays an important role in biodegradation of pollutant compounds in the environment. They are ubiquitous in the environment and are responsible for most of the recycling of carbon, nitrogen, sulfur, phosphorous and other minerals. Bacteria are the primary group responsible for the degradation of organic pollutant in the environment. Yeasts and fungi are less important, and microalgae and protozoa appear to be rarely involved in the degradation of these pollutant component. Fungi like bacteria metabolized organic compounds but do not compete

well in most engineered remediation system (Eweis, 1998). In well aerated soils, both fungi and bacteria plays an important role in degradation process but when poorly aerated conditions applied, bacteria usually will be responsible for the biological and chemical changes (Alexander, 1991). Hydrocarbon-degrading microorganisms are widely distributed in marine, freshwater, and soil ecosystems (Atlas and Bartha, 1973). The ability to isolate high numbers of certain oil-degrading microorganisms from an environment is commonly taken as evidence that those organisms are the active degraders of that environment (Okerentugba and Ezeronye, 2003).

2.5.1.1 Bacteria

Bacteria are a large group of unicellular microorganism, typically a few micrometers in length. Bacteria have a wide range of shapes ranging from spheres to rods and spirals. They are the most abundant group of organism present in soil and are an extremely diverse group of organism with widely varying morphological, ecological and physiological properties and are the primary degraders of natural and xenobiotic organic compounds (Golovleva, 1997) found in soils (Eweis *et al*., 1998). Because of their diversity, bacteria are usually found in heterogeneous communities. The most common genera of bacteria in soils are *Pseudomonas, Arthrobacter, Achromobacter, Micrococcus, Vibro, Acinetobacter, Brevibacterium, Corynebacterium*, and *Flavobacterium* (Eweis *et al*., 1998), Ojomu *et al.* (2005) and Adenipekun and Fasidi (2005) reported an extensive body of knowledge on mineralization or degradation of hydrocarbons by microorganism, most of these microorganism are of bacteria that have been characterized and classified using cultural, biochemical and molecular techniques. The capability of native bacterial

population to mineralize petroleum hydrocarbons in waste water was reported by Ojo (2006). Similarly, *Pseudomonas aeruginosa* demonstrated extensive degradation rate on crude oil with evidence of significant decrease of major oil component peak. Bola *et al*. (2006) reported *Pseudomonas*, *Bacillus, Alcaligenes* and *Citrobacter* were isolated from the core bitumen while the predominant species were mainly *Pseudomonas* species. All of these isolates were able to grow on crude petroleum as the sole source of carbon and energy when screened for hydrocarbon utilization. Microbial degradation of hydrocarbons often leads to production of organic acids and other metabolic products (Nwachukwu and Ugoji, 1995; Okpokwasili and James, 1995). Thus, the acids probably produced account for the reduction in pH levels. Some of the bacteria capable of producing endospores in extreme environment. Endospores are temporary conditions and the cells will revert to vegetative state as soon as the condition pervert.

2.5.1.2 Yeast

Yeast is eukaryotic microorganisms classified in the fungi kingdom. Currently there are about 1,500 species being described (Kurtzman and Fell, 2006) and they dominate the fungal diversity in the oceans. Yeast reproduces through the formation of budding or by binary fission. Their size can be varying depending on the species typically around 3 to 4 μm in diameter. Comparing to bacteria, most of the yeast do not contribute much in the field of bioremediation. Yeast was important in the field of alcohol beverage, ethanol production and food industry. But recently, some yeast was found to have potential application in bioremediation. *Yarrowia lipolytica* is known to degrade palm oil mill effluent and other hydrocarbons such as alkanes, fatty acids, fats and oils (Oswal *et al*., 2002; Fickers *et al*., 2005). *Yarrowia*

lipolytica also know to be the best degrader utilizing 78% of the aliphatic fraction comparing to other *Candida* species (Zinzarde and Pant, 2002). *Candida* species that have been described that contributed to the bioremediation industry were as *Candida parapsilosis*, *C. albicans*, *C. guilliermondii*, *C. tropicalis* and *C. intermedia* (Zinzarde and Pant, 2002).

Evaluation of hydrocarbon degrading potentials and emulsifying activities indicated that biosurfactant was one of the compounds that can be produced from the organism itself in accelerating bioremediation process. These microbial surfactants are environmentally friendly with amazing properties and spectrum of applications. Two newly isolated and promising yeast strains, *Saccharomyces cerevisiae* and *Candida albicans* were able to grow effectively on crude oil and diesel as sources of carbon and energy with the production of biosurfactant (Matthew *et al.,* 2008).

2.5.1.3 Fungi

Fungus is a member of the Kingdom Fungi. This eukaryotic organism is of monophyletic group, also called *Eumycota,* which is phylogenetically distinct from morphologically similar slime molds and water molds. The fungi are heterotrophic organisms possessing a chitinous cell wall, with the majority of fungal species growing as multicellular filaments called hyphae forming a mycelium. Some fungal species also grow as single cells. Sexual and asexual reproductions of fungi are commonly via spores, often produced on specialized structures or in fruiting bodies. Fungi use organic compounds for growth and energy. Inorganic contaminants such as nitrogen, phosphorus and other nutrients are incorporated into cell tissue in stoichiometric amounts (Eweis *et al*., 1998).

Relative to bacteria, fungi are less numerous, grow at considerably low rates and do not compete well in most engineered process environments. Additionally, metabolic processes of fungi are less diverse than bacteria. Fungi tend to be more acid-tolerant than bacteria and more sensitive to moisture content (Eweis *et al*., 1998). Certain fungi particularly white rot fungi can degrade insecticides, herbicides, pentachlorophenol, creosote, coal tars and heavy fuels and turn them into carbon dioxide, water and basic elements. Fungi have been shown to biomineralize uranium oxides, suggesting they may have application in the bioremediation of radioactively polluted sites. Kenneth (1995) reported ligninolytic fungi could help to accomplish the partial degradation of numerous aromatic organopollutants. Their ability to degrade polycyclic aromatic hydrocarbons (PAHs) is particularly interesting because eukaryotes were previously considered to be unable to cleave fused-ring aromatics. Extracellular peroxidases of these fungi are responsible for the initial oxidation of PAHs. *Phanerochaete chrysosporium*, a white rot *basidiomycetous* fungus is found to have potential in hazardous organic compounds treatment by producing extracellular peroxidases. However, the use of this species is limited when nitrogen source is low because the peroxidase is not produced (Eweis *et al.,* 1998).

2.5.1.4 Other microorganisms

Algae is one of the player but less important in the bioremediation process. Algae have been used in the bioremediation of aquatic system either by bioaccumulation of hydrophobic compounds in their lipids followed by harvesting of algal biomass or by degradation in the presence of sunlight (Okkeley and Deason, 1976; Matsumura and Esaac, 1979). Algae are sometimes used in nutrient removal but often are