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ISOLATION AND PHYLOGENETIC IDENTIFICATION OF COPPER-RESISTANT BACTERIA WITH HYDROCARBON DEGRADING ABILITY

LEE YAN LONG

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Lee Yan Long	*Dr. Wong Kok Kee*
I13002968	(SUPERVISOR)
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ABSTRACT

Two bacteria isolates (4M5 and A4) with hydrocarbon-degrading properties was subjected to copper (Cu) toxicity test at 2.0 mg/L, which is 10x higher than the permissive level for Cu in industrial effluents according to Malaysian Environmental Quality Regulations. Observation showed that isolate 4M5 was able to survive Cu at tested concentration ($p<0.05$) whereas A4 failed to survive ($p<0.05$). Identification test through Gram staining showed isolate 4M5 was Gram-negative, rod-shaped bacterium, whereas isolate A4 was Gram positive, rod-shaped bacterium. DNA of both bacteria isolates was extracted successfully and amplification of 16s rDNA region of the DNA using Polymerase Chain Reaction (PCR) yielded a band size of 800 bp. Result of the PCR product sequencing and BLAST analysis was used to construct a phylogenetic tree successfully, in which isolate 4M5 was identified as *Pseudomonas aeruginosa*, whereas isolate A4 was found to be *Bacillus subtilis*. Bacterial Adhesion Towards Hydrocarbon (BATH) Assay using isolate 4M5 showed negative result on production of biosurfactant ($p>0.05$) when exposed to Cu at 2.0 mg/L. Study on the biodegradation of crude oil co-contaminated by 2.0 mg/L of Cu showed increase in growth of isolate 4M5 by 10-fold ($p<0.05$) and 67% reduction of crude oil, indicating isolate *P. aeruginosa* 4M5 was able to biodegrade crude oil even in the presence of Cu at environmentally high level. This suggest the potential use of *P. aeruginosa* 4M5 in bioremediation strategy to clean up crude oil waste found in mixed industrial effluent.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
ATSDR	Agency of Toxic Substances and Disease Registry
BLAST	Basic Local Alignment Search Tool
Cu	Copper
$CuSO_4$	Copper(II) sulfate
CFU/mL	Colony-forming units per millilitre
DNA	Deoxyribonucleic Acid
EBI	European Bioinformatics Institute
EPA	Environmental Protection Agency
ITOPF	International Tanker Owners Pollution Federation Limited
L	Litre
MEGA	Molecular Evolutionary Genetic Analysis
mg/L	Milligrams per litre
min	minutes
mL	Millilitre
NaCl	Sodium chloride
NCBI	National Institute of Biotechnology Information
NOAA	National Oceanic and Atmospheric Administration
nm	Nanometers
OD	Optical density
PAHs	Polycyclic aromatic hydrocarbon
Pb	Lead
PCR	Polymerase Chain Reaction
P_{1B}-type ATPases	Large family of membrane proteins in plasma membrane

ROS	Reactive Oxygen Species
Rpm	Rounds per minute
TAE	Tris-acetate EDTA
U	Conversion of 1 micro-mole of substrate per minute

CHAPTER 1

INTRODUCTION

Following the advancement of technology and industry, crude oil has become the primary source of energy for daily industrial activities. Thus, the exportation and importation of Petrochemical industry, being a multimillion corporation for mining petroleum and exporting it to different countries, accidental spills along the transportation that causes pollution are therefore, inevitable. In 2016 itself, multiple incidents of oil spills has been recorded by The International Tanker Owners Pollution Federation Limited (ITOPF) and the major incident involves an oil spill of more than 700 tonnes by cargoes of gasoline and diesel in Gulf of Mexico (ITOPF, 2017). Crude oil composed of hydrocarbons, heavy metals and nitrogen compounds (World Ocean Review, n.d.) are known to be carcinogenic and neurogenic to organisms (Das & Chandran, 2011). Therefore, cleaning up crude oil pollution is very crucial in order to prevent any further damage to the environment. Malaysia, being the top few countries that mines petroleum is also susceptible to oil spills.

One of the numerous method used to clean up contamination due to crude oil hydrocarbon is bioremediation. Bioremediation uses bacteria that live under such natural habitat to degrade the crude oil hydrocarbon and consumes it as carbon energy source (United States Environmental Protection Agency, 2001). Many bacterial strains primarily from the genera of *Alcanivorax*, *Marinobacter*, *Pseudomonas*, and *Acinetobacter* have been reported having the ability to degrade hydrocarbons (Kostka et al., 2011). However, under the presence of co-contaminants such as heavy metals, which is found in hydrocarbon-associated environment the effectiveness of the degradation by bacteria is compromised (Wong et al., 2013).

According to Abioye (2011), co-contamination of heavy metals in industrial discharge and oil rigs are commonly found and significant amount of heavy metals can be detected in crude oil as reported by Sainbayar, Monkhoobor, and Avid (2012). Amor, Kennes, and Veiga (2001) reported that the type and concentration of heavy metals inhibited bacterial growth.

Wong, Quilty, and Surif (2013), further reported that heavy metals such as Cu and Pb inhibited the hydrocarbon-degrading enzymes, thus hampering the biodegradation process. Therefore, the objective of this experiment is:

1. to screen and isolate bacteria which is able to biodegrade hydrocarbons under the presence of copper (Cu), and
2. to identify the bacteria strain via phylogenetic analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 CRUDE OIL AND SOURCE OF CONTAMINATION

Crude oil is a complex mixture consisting of chemical components that are organic and inorganic which heavy metals being one of the inorganic components in the matrix (Sainbayar et al., 2012). 90% of the weight of crude oil is mainly made up of hydrocarbons (aromatic, aliphatic and alicyclic) and the remaining is made up of non-hydrocarbons compounds such as sulphur, oxygen and small amounts of heavy metals (Salleh et al., 2003; TOXNET NIH, 2016). **Table 2.1** shows the elemental composition of crude oil.

Table 2.1 The compositional elements found in crude oil

Composition of crude oil	Percentage (%)
Carbon	83-87
Hydrogen	10-14
Nitrogen	0.1-2
Oxygen	0.1-1.5
Sulphur	0.5-6
Heavy metals	<0.1

Source: Adapted from (Institute of Electrical and Electronics Engineers (IEEE), 2017).

Extensive consumption of crude oil in various industries, exploitation and transportation leads to soil and water contamination (Coleman et al., 2003). According to National Oceanic and Atmospheric Administration (NOAA) (2015), it was reported that 37% crude oil contaminations caused by discharges from ships and land-based sources can be traced back to industrial effluents (National Research Council, 2003), as shown in **Figure 2.1**.

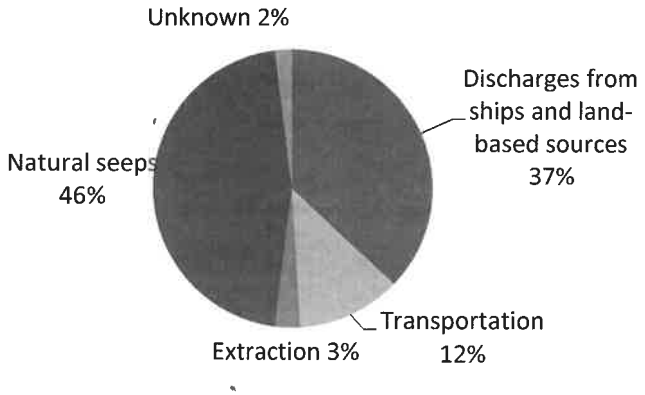

Figure 2.1 Sources of oil released into the marine environment that caused oil contamination. Adapted from NOAA 2015.

2.2 TOXICITY OF CRUDE OIL

Polycyclic aromatic hydrocarbons (PAHs) are one of the components which is mainly found in crude oil and is the component that allows crude oil to exerts toxicity (Olsen et al., 2013). PAHs carries toxigenic, carcinogenic and mutagenic properties (Bojakowska & Sokolowska, 2001) that causes increased risk in developing cancer (Boström et al., 2002). According to Pampanin and Sydnes (2013), one of the 16 PAHs which is found to be in highest concentration in crude oil is naphthalene following phenanthrene and fluorene. **Table 2.2** shows the minimum, maximum and mean volume of PAHs in 48 different crude oil tested.

Table 2.2 Different types of PAH and volume in 48 crude oils

Crude oil		mg/kg	
PAH	Min.	Max.	Mean
Naphthalene	1.2	3700	427
Acenaphthene	0	58	11.10
Acenaphthylene	0	0	0
Fluorene	1.4	380	70.34
Anthracene	0	17	4.3
Phenanthrene	0	400	146
Fluoranthene	0	15	1.98
Pyrene	0	9.2	-
Benzo[a]anthracene	0	16	2.88
Chrysene	4	120	30.36
Benzo[b]fluoranthene	0	14	4.08
Benzo[k]fluoranthene	0	1.3	0.07
Benzo[a]pyrene	0	7.7	1.50
Dibenz[a,h]anthracene	0	7.7	1.25
Benzo[g,h,j]perylene	0	1.7	0.08
Indeno[1,2,3-cd]pyrene	0	1.7	0.08

Source: Adapted from Polycyclic Aromatic Hydrocarbons a Constituent of Petroleum: Presence and Influence in the Aquatic Environment; (Pampanin & Sydnes, 2013).

One of the main characteristics of PAHs is low aqueous solubility, and thus highly hydrophobic (Abdel-Shafy & Mansour, 2016).The degree of hydrophobicity of PAHs depends on the number of rings that the molecule contains, the higher the number of rings, the more lipophilic it becomes (Abdel-Shafy & Mansour, 2016). Therefore, it tends to accumulate in membrane lipids and affects the saturation of fatty acids localized in the plasma membrane (Salar et al., 2014), and in turn disrupts the permeability and fluidity of the plasma membranes (Cerik et al., 2003) that leads to cell death. A study involved exposing zebrafish to PAHs was also reported to decrease rate of eggs reproduction, whereby the