ISOLATION AND IDENTIFICATION OF LIPOLYTIC BACTERIA FROM GALING RIVER WATER KUANTAN TO DEGRADE WASTE COOKING OIL

MUPIT DATUSAHLAN

MASTER OF SCIENCE

(BIOTECHNOLOGY)

UNIVERSITI MALAYSIA PAHANG

ISOLATION AND IDENTIFICATION OF LIPOLYTIC BACTERIA FROM GALING RIVER WATER KUANTAN TO DEGRADE WASTE COOKING OIL

MUPIT DATUSAHLAN

Thesis submitted in fulfillment of the requirements
For the award of the degree of
Master of Science (BIOTECHNOLOGY)

Faculty of Industrial Science and Technology
UNIVERSITI MALAYSIA PAHANG

SEPTEMBER 2015

UNIVERSITI MALAYSIA PAHANG

DECLARTION OF THESIS AND COPYRIGHT

Author's full name : MUPIT DATUSAHLAN

Date of birth : 30 OCTOBER 1988

Title : ISOLATION AND IDENTIFICATION OF LIPOLYTIC BACTERIA FROM GALING RIVER WATER KUANTAN TO DEGRADE WASTE COOKING OIL

Academic Session : 2014/2015

I declare that this thesis is classified as:

☐ CONFIDENTIAL (Contains confidential information under the Official Secret Act 1972)*

☐ RESTRICTED (Contains restriction information as specified by the organization where research was done)*

☐ OPEN ACCESS I agree that my thesis to be published as online open access (Full text)

I acknowledge that University Malaysia Pahang reserves the right as follows:

1. The Thesis is the property of University Malaysia Pahang
2. The Library of University Malaysia Pahang has the right to make copies for the purpose of research only.
3. The Library has the right to make copies of the thesis for academic exchange.

Certified By:

MUPIT DATUSAHLAN
Passport Number: A1004703
Date:

DR. WAN FAIZAL BIN WAN ISHAK
IC Number: 72 0317 035339
Date:

NOTES : *If the thesis is CONFIDENTIAL or RESTRICTED, please attach with the letter from the organization with period and reasons for confidentiality or restriction.

SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality of the award of the degree of Master of Science in Biotechnology.

Signature :
Name of Supervisor : DR. WAN MOHD FAIZAL BIN WAN ISHAK
Position : SENIOR LECTURER
Date :

Signature :
Name of Co-Supervisor : DR. ESSAM ABDULLATIF MAKKY
Position : SENIOR LECTURER
Date :

STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for the quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for the award of any other degree.

Signature :
Name : MUPIT DATUSAHLAN
ID Number : MKT11002
Date :

Dedicated to my wife, my son, my parents, my brothers, my sister and friends, who gave me everlasting inspiration, never-ending encouragements and priceless support towards the success of this study.

ACKNOWLEDGEMENTS

My opportunity gratitude to Allah S.W.T, the Almighty for giving me this gave the great chance to enhance my knowledge and to complete this research. May the peace and blessings be upon prophet Muhammad (SAW).

I would like to take this precious moment to express my sincere appreciation and grateful appreciation to my supervisor, Dr. Wan Mohd Faizal bin Wan Ishak, and Co-Supervisor Dr. Essam Abdullatif Makky this support, advice and for their germinal ideas and mentorship in my research.

I am grateful to the Faculty of Industrial Sciences and Technology, University Malaysia Pahang for the support of my research and study.

I would like to thank all the members of Postgraduate and Technical Staff of Faculty of Industrial Sciences and Technology, University Malaysia Pahang for their assistance and sharing of their expertise to me.

Especially, I would like also to address my unlimited thanks to my wife Lia Mardiati and my son Ghalib Ahnaf Daffa, my parents Basuki (Alm) and Sofiatun, to my sisters and brothers Miftahul Jannah, Nurul Hidayati, Elik Muflikah, sukirman, wijianto, kusdiono, Lianawati, Putri Ayu Widyaningsih, Dwi Yuli N.S, Dea Merly Kirnasari, Putra Bagus Nugroho, Angga Saputra (wilujeng), Vira and Arif Wicaksono, My friends in UMP ibu Noor Hindryawati S.pd. M.Si, Irma Nurfitri S.pd, Ainul Hizriadi S.kom, Haniif prasetyawan S.T, Pak Nugroho Dewayanto Ms.c. Ph.d, Dr. Anwarudin Hisham, Dr. Luhur Bayu Adji, Dr. Rama Yusvana, Ibu Ita Lutfiana, Dr. Gigih Prihandoko, Pak Budi santoso, Pak Suhirman M.kom, Pak Sutarman M.kom, Makson Rivai, mas ardi Wicaaksono, Mohammad Hisyam Abdullah, Mohd Lokman and kak Marni, for support, their patience, love and trust in during my study in Universiti Malaysia Pahang.

ABSTRACT

Galing River is located in the center of the Kuantan city. The river was polluted by various waste and categorised as Class IV by Department of Environment. One of pollution sources is identifying came from waste cooking oil. The objective of this study is to isolate and identify of lipolytic bacteria potential, which originated from the location of the downstream Vistana hotel at the Galing River water. The ability of lipolytic bacteria in degradation of waste cooking oil is also investigated. The potential of lipolytic bacteria investigation is by using Rhodamine B. MacConKey agar is used to identify the presence of lipolytic bacteria. RapID ONE test used for the selected media, which will identify the bacteria strain name, while for a final identification is using PCR and DNA analysis. To determine the lipolytic ability of the bacteria in degrading the waste cooking oil, FTIR and gravimetric analysis are used. Most of the pollutants appear with the WQI value of 32.61. Oil and Grease value at the location M4 is 14 mg / L, while the lowest level on M1 with a value of 2 mg /L, while M2 and M3 respectively 4 mg /L and 7 mg /L. There are four bacterial strains, which had been isolated from M4 location, i.e.: *Providencia stuartii, Bacillus pimilus*, *Exiquobacterium* sp., and *Bacillus antracis*. The identified bacteria will be used to degrade waste cooking oil (WCO). The variation of degradation time and concentration of bacteria was observed. Measurable objective of the lipolytic bacteria capability to degrade the waste cooking oil (WCO) source in batch production based on the optical density and gravimetric data, and the rate of degradation of waste cooking oil (WCO) using FTIR. Based on that analysis, it is concluded that gravimetric analysis has a maximum value degradation of 91.39% in 100 ml variation bacteria and 4% waste cooking oil (WCO). While the minimum value of the variation of 50 µl bacteria and 1% waste cooking oil (WCO) degradation 17.08%. It's based on a variety of media (50, 75 and 100 µl) of bacteria and 1-4% waste cooking oil (WCO). The trend increase in from 1-4% with variation bacterial of (50, 75 and 100 µl). While the degradation of waste cooking oil (WCO) using FTIR was obtained the new climax that were observed at 721 and 869 cm-1 was shown the presence of aromatic compound due to a benzene ring. FTIR provides a quick and accurate way to evaluate the structural changes from the waste cooking oil (WCO) that degraded cause by of the bacteria. As the results of this study, it can be concluded that the isolation of bacteria derived from Galing river can be used to degrade the waste cooking oil (WCO).

ABSTRAK

Sungai Galing terletak di pusat bandar Kuantan. Sungai itu dicemari oleh pelbagai sisa dan dikategorikan dalam Kelas IV oleh Jabatan Alam Sekitar. Salah satu punca pencemaran datang dari sisa minyak masak. Objektif kajian ini adalah untuk mengasingkan dan mengenal pasti bakteria lipolytic berpotensi, yang berasal dari hiliran air Sungai Galing berdekatan Hotel Vistana. Keupayaan bakteria lipolytic dalam degradasi sisa minyak masak juga disiasat. Potensi bakteria lipolytic dikaji dengan menggunakan Rhodamine B. Agar MacConkey digunakan untuk mengenal pasti kehadiran bakteria lipolytic. Ujian Rapid ONE digunakan untuk media terpilih, yang akan mengenal pasti nama strain bakteria, manakala bagi peringkat akhir pengenalpastian dengan menggunakan PCR dan analisis DNA. Untuk menentukan keupayaan lipolytic bakteria dalam penguraian sisa minyak masak, FTIR dan analisis gravimetrik digunakan. Kebanyakan bahan pencemar muncul dengan nilai WQI sebanyak 32.61. Nilai minyak dan gris di lokasi M4 ialah 14 mg/L, manakala tahap paling rendah hanya di M1 dengan nilai sebanyak 2 mg/L, manakala M2 dan M3 masing-masing 4 mg/L dan 7 mg/L. Terdapat empat jenis bakteria, yang telah diasingkan daripada lokasi M4, iaitu: *Providencia stuartii, Bacillus pimilus, Exiquobacterium* sp, dan *antracis Bacillus.* Bakteria yang dikenal pasti akan digunakan untuk menguraikan sisa minyak masak (WCO). Perubahan masa kemerosotan dan kepekatan bakteria diperhatikan. Objektif yang boleh diukur ialah keupayaan bakteria lipolytic menguraikan sumber sisa minyak masak (WCO) dalam satu kelompok pengeluaran berdasarkan ketumpatan optik dan data gravimetrik dan kadar penguraian sisa minyak masak (WCO) menggunakan FTIR. Berdasarkan analisis, dapat disimpulkan bahawa analisis gravimetrik mempunyai nilai maksimum kemerosotan 91.39% dalam 100 ml variasi bakteria dan 4% sisa minyak masak (WCO). Manakala nilai minimum degradasi bagi variasi 50 µl bakteria dan 1% sisa minyak masak (WCO) ialah 17.08%. Ia berdasarkan pelbagai media (50, 75 dan 100 µl) bakteria dan 1-4% sisa minyak masak (WCO). Trend ini meningkat pada 1-4% dengan variasi bakteria (50, 75 dan 100 µl). Manakala, penguraian sisa minyak masak (WCO) menggunakan FTIR telah diperolehi menunjukkan kemuncak baru diperhatikan pada 721 dan 869 cm-1 kerana kehadiran sebatian aromatik benzena. FTIR menyediakan cara yang cepat dan tepat untuk menilai perubahan struktur daripada sisa minyak masak (WCO) yang diurai disebabkan oleh bakteria. Daripada hasil kajian ini, dapat disimpulkan bahawa pengasingan bakteria yang diperoleh daripada Sungai Galing boleh digunakan untuk menguraikan sisa minyak masak (WCO).

TABLE OF CONTENT

LIST OF TABLE

LIST OF FIGURES

LIST OF SYMBOLS

gr	Gram
%	Percentage
µl	Microliter
h	Hours
L	Liter
R	Rotation
M	Molar or Mole
F	Fahrenheit
min	Minutes
S	Second
ml	Milliliters
nm	Nanometer
ºC	Celsius Degree
pH	Potential hydrogen /Hydrogen Ion Concentration
V	Volume
n	nano
w	Weight
M1	Street Industrial Semambu 4
M2	Drain Lorong Seri Setali 60
M3	Drain Street Galing 33
M4	Downstream Vistana Hotel

LIST OF ABBREVIATIONS

ABD	Acetylbutanediol
API	Analytical Profile Index
APHA	American Public Health Association
BLAST	Basic Local Alignment Search Tool
BOD	Biological Oxygen Demand
CFU	Colony Forming Unit
COD	Chemical Oxygen Demand
DAS	Design Absorb System
DMPD	Dimethyl-p-Phenylenediamine
DNA	Deoxyribonucleic Acid
DO	Dissolve Oxygen
DOE	Department of Environment
DTP	Dissolve Total Phosphorus
EPA	Environmental Protection Administration
ESBL	Extended Spectrum Beta Lactamase
FAO	Food and Agriculture Organization
GAC	Granular Activated Carbon
INWQS	Interim National Water Quality Standard
LB	Luria Broth
MPK	Majlis Perbandaran Kuantan
MPN	Most Probable Number
NA	Nutrient Agar
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
OD	Optical Density
O & G	Oil and Grease
PAC	Powdered Activated Carbon
PCR	Polymerase Chain Reaction
PDLA	Port Dickson Local Authority
PE	Population Equivalent

ppb	Part Per Billion
RNA	Ribonucleic Acid
ROD	Remaining Oil Degradation
RPI	River Pollution Index
rpm	Rotation Per Minute
rRNA	Ribosomal Ribonucleic Acid
SMSs	Stone Mineral Salt Solution
SMSSe	Stone Mineral Salt Solution Enrich
TDS	Total Dissolve Solid
TFR	Trickle Flow Reactor
TMPD	Tetramethyl-p-Phenylenediamine
TN	Total Nitrogen
TOC	Total Organic Carbon
TP	Total Phosphorus
TSS	Total Suspended Solid
USEPA	United States Environmental Protection Agency
WCO	Waste Cooking Oil
WHO	World Health Organization
WPCO	Waste Palm Cooking Oil
WQI	Water Quality Index
WQS	Water Quality Standard

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

Water is a valuable natural resource for the survival of human beings. Surface and ground water are very distinct sources of water. Humans have used water systems for numerous purposes such as for drinking, irrigation, fisheries, industrial processes, transportation and domestic waste disposal. With such a high dependence on water, increasing of urbanization, agricultural and industrial practices caused adverse effects on both surface and ground water, thus rapidly decreasing the water quality (Vazquez et al., 2003). This quality refers to the physical, chemical and biological characteristics of water. The term "water quality" is intimately related to water pollution. Water pollution is emerging as a threat to all humanity. The problem of environmental pollution has been generally due to the ever-increasing industrialization, urbanization, high density of population and the unplanned introduction of plant, which often proves to be hazardous to human health. A wide range of human activities in agroindustry, livestock, tourism, and other activities also disposes waste. These disposed materials contain a high organic compound of lipid, carbohydrate, and protein. Without proper treatment, these materials are normally associated with the cause of health problems and environmental pollution (Kalyani et al., 2009).

Galing River is located in Kuantan City, Pahang, Malaysia. Most activities by human near Galing River are housing estate, workshop, hotel, restaurant and petrochemical industry (Dhall et al., 2012; and Hamzah et al., 2012). In another study, the Galing River was found to be polluted and considered as the major river in Pahang state as it provides water for industries, agriculture, and human consumption. The

Galing river is polluted by both conventional pollutants (heavy metal, nitrate, phosphate, pesticides) and also polluted by Fat, oil and grease which cause major problems to drains and sewers. When they are disposed from down kitchen sinks or drains they cause blockages; when they enter rainwater pipes or gullies they cause pollution in streams and rivers. In addition to micro-pollutants from industries around Galing river (Pohan, 2010). In another source, it is expected that isolated bacteria from Galing river sludge can be local bacteria resistant to many pollutants. The bacteria can be utilized to degrade water pollutants in river bank of Galing and its surroundings due to industrial activities (Abdo et al., 2010).

The oily wastewater, especially from the oil field, housing area, workshop, industry, and restaurant, has posed a great hazard for terrestrial and river ecosystems (Kanu and Achi., 2011). The traditional treatment of oily wastewater, such as containment and collection using floating booms, adsorption by natural or synthetic materials, etc., cannot degrade the crude oil thoroughly (Zhang et al., 2005). So far, bacteria biodegradation suggests an effective method. During biodegraded lipolytic bacteria, crude oil is used as an organic carbon source by a microbial process, resulting in the breakdown of crude oil components to low molecular weight compounds.

Oil and its derivative, from oil manufacturing plants and restaurants have received much attention because of their widespread use. Disposal of this wastewater into environment causes serious problems due to its high oil content, chemical oxygen demand (COD) and color. Microbial degradation of oil wastewater is a concern in recent years. A variety of microorganisms such as bacteria, molds, and yeasts, have been shown to be capable of completely degrading oil wastewater (Ammar et al. 2005; Dhouib et al. 2006; Erguder et al. 2000; Ettayebi et al. 2003; Kissi et al. 2001). Zhang et al., (2005) utilized oil as a substrate for biomass production of yeast isolate, and Ban-Weiss et al. (2011) reported using oil mill wastewater (OM)-based on media producing citric acid, the phenolic compounds of OWM were decreased simultaneously and a remarkable decolorization was observed.

Biological treatment of waste cooking oil and oil and grease from food wastewater by bacteria lipolytic has been studied (Wu et al., 2006). With the concentration of oil 2000

mg/L, the removal rates of waste cooking oil and grease from food wastewater by free cells attained 93.30% and 85.08% under the optimum conditions for 50 h, respectively. Based on the description above, in this study there is a need conduct lipolytic bacteria that have the ability to degrade waste cooking oil. This is in tandem wide previous research such as *Bacillus* sp., *Clostridium* sp., *Staphylococcus, Mycobacterium, Escherichia, Providences, Erwinia, Proteus vulgaris, Acinetobacter radioresistent* and *Pseudomonas aeruginosa* Hamzah et al., 2012, has referenced. This study is to added by, whether the insulation lipolytic bacteria from the Galing river from contaminated sources which have the same types and the ability to degrade the waste cooking oil that pollutes rivers.

1.2 PROBLEM STATEMENT

The purpose of water treatment is to provide clean water that does not contain objectionable taste, odor, or color; it also provides adequate quantities of water for domestic, commercial, industrial and fire protection needs. All water produced in public water systems is required to achieve clean water quality, even though only about 1% of water produced is used for drinking and cooking. Through water treatment, clean and treated water should be accessible to all people, not only for an urban population where all the facilities and amenities are available but also for people who live in remote and rural areas.

Galing River is currently classified in class III and IV, and will be upgraded to class II. The pollutants that contaminate the river come from industrial waste, domestic waste, hospitality industry (restaurant, hotel, etc.), agriculture, land clearing, waste of the workshop, and also polluted Fat, oil and grease and waste cooking oil (WCO) which causes major problems to drains and sewers. When they are disposed of down kitchen sinks or drains they cause blockages; when they enter rainwater pipes or gullies they cause pollution in streams and rivers. Besides the various other activities that cause pollution the river. Based on the mentioned problems, the treatment of source wastewater pollutant it needs to be treated by using bioremediation by utilizing bacteria that live in the source waste water river. These problems in the Galing river can be overcome by improving the river water quality index.

Studies on Galing river water has been done by several sources elsewhere. Have the isolation and identification of lipolytic bacteria that have the ability to degrade the pollutant source of waste cooking oils and oil and grease should be investigated. Because this type of waste is very difficult to decompose in water. This is due to the bacteria that live in the location of the pollution which is estimated to have the ability to reduce the source of the waste itself. Then isolation of bacteria from the polluted river water will source test the ability of bacteria to degrade the waste cooking oil with the optimization of the growth of bacteria, variation bacterial and source of feed. The question is whether the bacteria from Galing river water can degrade the pollutant source.

1.3 RESEARCH OBJECTIVES

Based on the problem statement above, the objectives of this study consist of:

1. To isolate and identify the potential of lipolytic bacteria from Galing River water.
2. To measure the ability of lipolytic bacteria in degrading waste cooking oil.

1.4 SCOPE OF STUDY

To achieve the above-mentioned objectives, the following scopes of study are designed:

1. Identifying the quality of Galing river water, namely pH, Temperature, Dissolved Oxygen (DO), Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Ammonical Nitrogen (AN), Total Suspended Solids (TSS), (Heavy metals: Iron), *E. coli*, Oil and Grease and classification of river water (Class I-V).

2. Identifying and isolating the existences of bacteria using gram straining and color plate disc to reduce sources of pollutant from waste cooking oil from Galing river water, and Characterization of the isolated bacteria using Gram straining, physiological study by microscope, and the determination of genus by PCR and DNA.

3. To study on the ability of the bacteria to degrade waste cooking oil with parameter conditions of waste cooking oil by 1-4% by weight and incubation time of 1 – 6 days in batch production.

CHAPTER 2

LITERATURE REVIEW

2.1 RIVER WATER POLLUTION

The definition of pollution refers to "any substance introduced into the environment that negatively has affected the usefulness of a resource or the addition of a matter by human activity to the environment". We must be more sensitive about everything that had happened in our environment. Pollutants may cause primary damage, with direct identifiable impact on the environment, or secondary damage in the form of minor perturbations in the delicate equilibrium of the biological food webs that are obvious only over long time periods. Generally, pollution means the introduction by man, directly and indirectly, of substances or energy into the environment resulting in such poisonous effects as harm to living resources, hazards to human health, harmful to land, soil, air and interruption of marine activities, including fishing, impairment of quality for use of sea water and reduction of amenities (Atlas, 1995; Bako et al., 2008; Abdo et al., 2010; Jamalludeen et al., 2009; and Chowdhuri et al., 2011). Environmental pollution is any discharge of material or energy into water, land, or air that causes or may cause acute (short-term) or chronic (long-term) detriment to the Earth's ecological balance or that lowers the quality of life (WHO, 2004). Environmental pollutants that enter water bodies from municipal, agricultural, and industrial sources may remain suspended in the water column, be taken up by aquatic biota, or settle on the bottom and become incorporated into the sediments (Berg et al., 2001; and Valdes et al., 2010).

2.2 SOURCES POLLUTANT IN WATER RIVER AND EFFECT OF WATER TO ENVIRONMENT

Wastewater is the spent water after houses, commercial establishments, industries, public institutions, and similar entities have used their waters for various purposes. It is synonymous with sewage, although sewage is more general term that refers to any polluted water (including waste-water), which may contain organic and inorganic substances, industrial wastes, domestic wastewater, industrial wastewater and agriculture wastewater, groundwater that happens to infiltrate and to mix with the contaminated water, storm runoff and other similar liquids. Certain sewage may not be a spent water or wastewater (Swannell et al., 1996; Sugai et al., 1997; and Simmons, 1991). There are two sources of contamination in polluted areas which are the source of water pollution and sediment contamination source.

2.2.1 Pollution in Water

Water is one of the essentials that supports all forms of plant and animal life (Vanloon and Duffy., 2005) and it is generally obtained from two principal natural sources; Surface water such as fresh water lakes, rivers, streams, etc. and Ground water such as borehole water and well water (Mendie, 2005; and McMurry and Fay, 2004) This is a reason. That's why water has often been described as 'the universal solvent" or "the liquid of life". Water is a vital for drinking, without water, no human, flora and fauna could survive, and it is used for maintaining personal hygiene. In recent years, water resources are under stress worldwide and one of the elements of this stress as due to men impact on water quality that constitutes a key problem especially in well-developed countries.

The definition of water pollution, mainly the alteration in the composition or condition of natural water either directly or indirectly as the result of human activities, which initiates modification of ecological systems, hazards to human health and impairs the subsequent water use. Much literature mentioned that heavy metals are the common waste products on increasing of anthropogenic activities and their emission often results in the pollution of the surrounding environment. Being discharged directly to the surface waters, constituents of industrial effluents creates a significant hazard to aquatic

ecosystems (Schwarzbauer and Ricking, 2010; Tusseau-Vuillemin et al., 2007; Focazio et al., 2008; Botalova and Schwarzbauer, 2011; and AL et al., 2006). The levels of certain trace elements in rivers, lakes and other water systems have been found to be moderate, very, and high as a result of industry discharges (Al-Masri et al., 2002; and Coker et al., 1995).

Water pollution has seriously affected many rivers, lakes, sea and even parts of the oceans. With the increasing technology in every sector that gives stress to the water resources and environment, clean water is becoming polluted by several men made activities, e.g. increasing of population growth, industrialization and deforestation, urbanization, increasing living standards and wide spheres of other human activities. That is among the reason it is of special interest to study the water pollution because this case is part of our life.

Sources of contamination of water pollution are as follows:

(i). Point source

a. Sewage and domestic wastes
b. Industrial effluents
c. Soap and Detergents

(ii). Non-point source

a. Agricultural Discharges
b. Pesticides and Fertilizer
c. Thermal Pollution

These pollutants are straight released by industrial plants and municipal sewage treatment plants, others come from polluted runoff in urban and agricultural areas and some as the result of historical contamination, which in turn increases the concentration of nutrients along with other wastes in the marine environment (Zhou et al., 2004; Hang et al., 2009; and Begum et al., 2009). For this reason, environmental monitoring has become recognized as being vitally important in detecting where insidious pollution is occurring. The pollutants involved and sources from which they came. Therefore, the influence of anthropogenic traced inputs on coastal areas needs further research. Moreover, the distribution of heavy metals from the river system through estuaries and coastal areas also need more understanding.

2.2.2 Pollution in sediment

Sediments are composed of all detritus, inorganic or organic particles eventually settling on the bottom of a body of water (Abul Kashem et al., 2007; Ankley et al., 1992; Bartolomeo et al., 2004; Leivouri, 1998; Maher and Aislabie, 1992; and Korfali and Jurdi, 2011). They originate from soil erosion and precipitation from chemical and biological processes in the water. Geologically speaking sediments are at the end of the path for natural and anthropogenic materials, which is the root of contaminated problems (Burton et al., 2004).

The U.S. Environmental Protection Agency (USEPA) identifies fluvial sediment as the single most extensive pollutant in rivers and streams, negatively affecting aquatic habitat, drinking water treatment processes, and recreation at rivers, lakes, and estuaries (USEPA, 1998). Sediment also is the principal conveyor of other pollutants, controlling the transfer, fate, and effect of the main contaminants (i.e., Toxics) (Chapman et al., 2002; and Allan et al., 2006). Fine-grained sediment is an intrinsic and vital component of river systems and plays a major role in the hydrological, geomorphologic, and ecological functioning of rivers (Walling and Fang, 2003). Even though sedimentation is a naturally-occurring phenomenon, anthropogenic activity has caused significant changes in the quantity and quality of fine-grained sediment within lentic and lotic systems (Walling and Fang, 2003; and Allan et al., 2006).

After the industrial revolution, point sources from mining, municipalities, industries and non-point sources from both agriculture and urban storm water runoff have accumulated in-water resources, settling to the bottom areas of water bodies (Nriagu, 1979). Contaminated sediments pose a risk to the environment in two basic mechanisms: (1) ecological risk to aquatic and piscivorous animals and (2) toxic risk on terrestrial habitat when contaminated area is dredged and placed on land (Khan et al., 2000).

In river Cape, surface waters and groundwater as well as sediments and soils will be affected by storm water discharge, which is an important contamination source for trace metals and polycyclic aromatic hydrocarbons (Renzt et al., 2011; and Brown

and Peake, 2006). The geomorphology and geochemistry of the water bodies and their catchment area determine which and how processes take place in the water bodies. The most important sources of this pollution are the burning of fossil fuels, mining and smelting of metalliferrous ores, metallurgical industries, municipal wastes, fertilizers, pesticides and sewage. Soil, at the interface between the atmosphere and the earth's crust, is open to inputs of heavy metals from many of these sources (Alloway and Ayres, 1997).

2.3 GLOBAL WASTE WATER RIVER MANAGEMENT

This widely used terminology refers to three levels of wastewater treatment: primary, secondary, and tertiary (or advanced). Primary (mechanical) treatment is designed to remove grease, suspended and floating solids from raw sewage. It includes screening to trap solid objects and sedimentation by gravity to remove suspended solids. This level is sometimes referred to as "mechanical treatment", although chemicals are often used to accelerate the sedimentation process. Primary treatment can reduce the BOD of the incoming wastewater by 20-30% and the total suspended solids by some 50-60%. Primary treatment is usually the first stage of wastewater treatment (Sugai et al., 1997; Pritchard et al., 1992; and Bragg et al., 1994). Many advanced wastewater treatment plants in industrialized countries have started with primary treatment, and have then added another treatment stage as wastewater load has grown, as the need for treatment has increased, and as resources have become available.

Secondary (biological) treatment removes the dissolved organic matter that escapes primary treatment. This is achieved by microbes consuming the organic matter as food, and converting it to carbon dioxide, water, and energy for their own growth and reproduction. The biological process is then followed by additional settling tanks (secondary sedimentation), to remove more of the suspended solids. About 85% of the suspended solids and BOD can be removed from a well running plant with secondary treatment (Atlas, 1995; and Ketan et al., 2009). Secondary treatment technologies include the basic activated sludge process, the variants of pond and constructed wetland systems, trickling filters and other forms of treatment which use biological activity to break down organic matter.

Tertiary treatment is simply an additional treatment beyond the secondary Tertiary treatment that can remove more than 99 percent of all the impurities from sewage, producing an effluent of almost drinking-water quality. The related technology can be very expensive, requiring a high level of technical know-how and well trained treatment plant operators, a steady energy supply, and chemicals and specific equipment which may not be readily available. An example of a typical tertiary treatment process is the modification of a conventional secondary treatment plant to remove additional phosphorus and nitrogen.

2.3.1 Waste Water Management in Malaysia

Malaysia has a population of 28.3 million based on the Report of Census 2010 by the Department of Statistics. The estimated volume of wastewater generated by municipal and industrial sectors is 2.97 billion cubic meters per year. The proportions of population equivalent (PE) served by the various sewerage systems are shown in Figure 2.1.

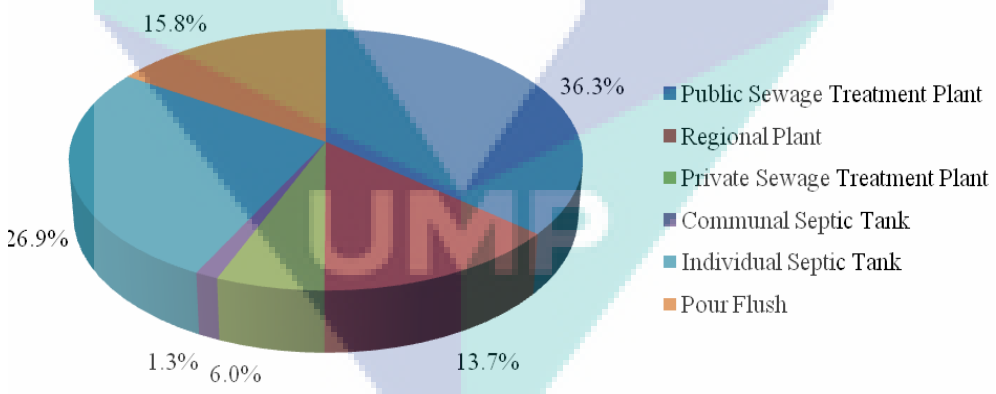


Figure 2.1: Proportions of population equivalent (PE) served by the various sewerage Systems

According to Indah Water Konsortium (the country's main sewerage operator), the dominant wastewater treatment types are preliminary (removal of rags, rubbish, grit, oil, grease), primary (removal of settleable and floatable materials), and secondary treatment (biological treatment to remove organic and suspended solids). At the moment, there is no plan to build tertiary treatment systems in

Malaysia. The focus has been providing a basic standard of preliminary, primary, and secondary treatment.

The major constraints to wastewater treatment faced by Malaysia in the present scenario there are, the low sewerage tariff is unable to support the high operation and maintenance costs. Noncompliance or mediocre compliance is mainly caused by high influence of oil and grease (O&G) discharged into serving industrial, commercial areas and food related business premises that do not have grease traps or do not maintain grease traps adequately. Other contributory factors are excessive discharge of soaps, detergents and other cleaning agents into the sewerage system. Sewerage services collection of operators is not conducive as it is unfortunate that many Malaysians fail to realize the importance of sewage management with regards to a safer environment. To curb this government has initiated a proposal to introduce joint billing for water supply and sewerage services. The dynamics of the sewerage industry where sewerage infrastructures are constructed by private developers and handed over to the public operator (for operations and maintenance) opens up the risk factor of quality being compromised, which would subsequently have an impact on the treatment processes and operations (Hamid and Baki, 2011).

2.3.2 Wastewater use/disposal

Currently there is no information on the area of food crops irrigated with wastewater as the Government of Malaysian has yet to have a policy on the reuse of wastewater whether treated or untreated, for irrigation. The average annual rainfall is more than 2,800mm and the estimated annual total surface water is 566 billion cubic meters, of which only 10% is readily available to satisfy the demands of agriculture, domestic, industry and power sectors (Mat et al., 2011) Although the volume of surface water is able to meet the demand of various ues and applications at the moment, there were few occasions in the past where the country had faced adverse water shortage due to extreme drought condition during the dry season (MOH, 2011).

It is mandatory for wastewater in urban areas and townships to be treated before discharged into surface waters. The quality of effluent from treatment plants is regulated by the Environmental Quality Act 1974 and its regulations such as the Environmental Quality (Sewage) Regulations 2009 and Environmental Quality (Industrial Effluent) Regulations 2009. In most rural areas, pour flush latrines are introduced. Therefore, there is no direct use of wastewater in the agriculture sector as treated wastewater effluent had undergone dilution when coming into contact with surface waters (DOE, 2011)

2.4 GALING RIVER IN KUANTAN MALAYSIA

Water bodies are defined as any significant accumulation of water occurring on Earth's surface. 'Body of water' refers to oceans, seas and lakes, but also smaller pools of water, like ponds, puddles or wetlands are included. Geographical features where water moves from one place to another, like rivers, streams and channels are not always considered bodies of water, but they can be included as geographical formations featuring water. River can be defined as having unidirectional flow, with generally short retention times measured in days rather than weeks. They have a relative high average flow velocity of between 0.1 and 1.0 ms^{-1}, although the discharge rate is highly variable over time, and is a function of climate and discharge a condition within the watershed. These features include the size, form and the geological characteristics of the basin and the climatic conditions which determine the quantities of water to be drained by the river network. Rivers drain watersheds of varying dimensions. As indicated in Table 2.1 this area is directly related to the river discharge and width.

Table 2.1: Classification of rivers based on discharge characteristic and the drainage area and river width

A River size	Average discharge (m^3s^{-1})	Drainage area (km^2)	River width (m)	Stream order
Very large rivers	>10,000	$>10^6$	>1,500	>10
Large rivers	1,000 – 10,000	100,000 - 10^6	800 – 1,500	7 to 11
Rivers	1,00 – 1000	10,000 – 100,000	200 – 800	6 to 9
Small Rivers	10 – 100	1,000 – 10,000	40 – 200	4 to 7
Streams	1 – 10	100 – 1000	8 – 40	3 to 6
Small Streams	0.1 – 1.0	10 – 100	1 – 8	2 to 5
Brooks	< 0.1	< 10	< 1	1 to 3

Rivers are highly important for both natural systems and human societies (Simmons, 1991; and Renzt et al., 2011). They form the physical environment, and are permanently changing it. Rivers and lakes are habitats for animal and plant species, and are suppliers of water and food. They fill transport functions, and they propose conditions for economic business development. Rivers are also energy sources. The shores of rivers and lakes give quality of life (waterfront development) and space for recreation in urban areas. River sediments are basic components of our environment as they provide nutrients for living organisms and serve as sinks for deleterious chemical species (Lasheen and Ammar, 2009; and Gonzalez-Macias et al., 2011).

Industrial activities have taken place on their banks for more than 100 years (Renzt et al., 2011; and Olofsson, 2002). With the advent of industrialization and increasing populations, the ranges of requirements for water have increased together with greater demands for higher quality water. Major river water users can be summarized as follows: sources of drinking water supply, irrigation, agricultural lands, industrial and municipal water supplies, industrial and municipal waste disposal, navigation, fishing, boating and body-contact recreation, Aesthetic value.

2.4.1 Location of Galing River

Galing River is a small stream situated in the Centre of Kuantan town. It is divided into two separate streams that are Galing Besar (Big) and Galing Kecil (Small). An estimation distance of these two streams is 7.7 km. Human activities are a major pollution contribution to this river where it flows through out the industrial area (Semambu), housing area and also commercial areas (City Center). Galing Besar is the main drainage system for the east side of Kuantan town. Major drainage systems of from all over the catchment area, including the Galing Kecil are flushed to the Galing Besar.

Galing River has been identified as the dirtiest river in Pahang in 2009. The river water quality is stated as Class IV which is only suitable for irrigation (Omar, 2010). The cause of this problem is industrial waste effluent, discarded solid waste, unplanned land use and sewage effluent from septic treatment pond that is not well-functioned.

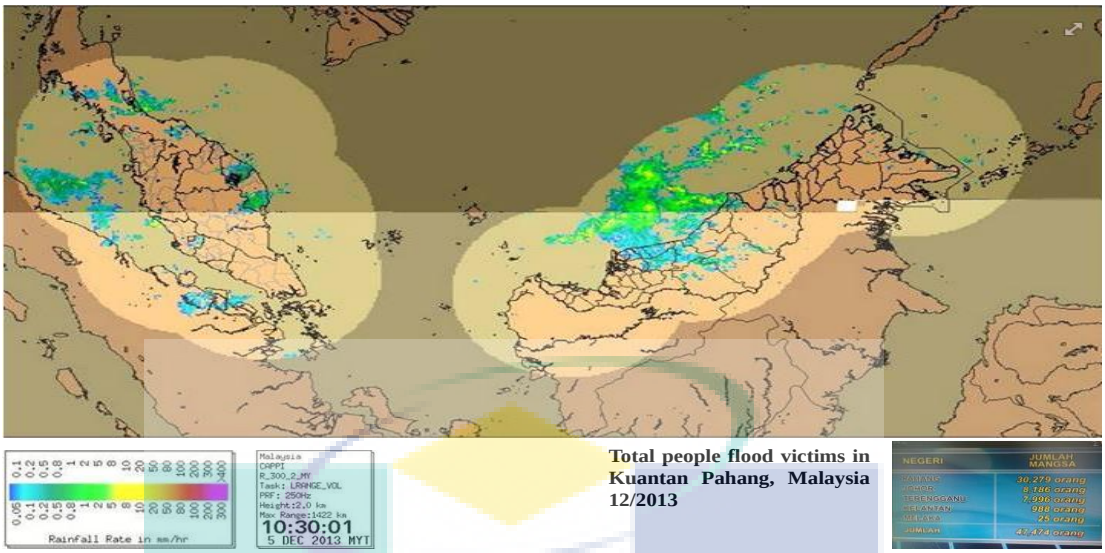


Figure 2.2: Land use Kuantan Pahang Malaysia, locations sampling and condition new weather extreme December 2013

Figure 2.3: Maps of the study area. The colored line on the left is Galing Besar while on the right is Galing Kecil.

2.4.2 Population and Issues Surrounding on Galing River

Table 2.2, shown the population surrounding Galing River as reported by Population and Housing Census of Malaysia 2010. From the Table shown, the highest population is in Area 9 with a total population of 6,293. Among the major issues and problems identified are the rapid development in the upper catchment and less effort in environmental preservation. Illegal dumping of solid waste from commercial areas, housing, villages, and other slum settlements, pesticides / chemicals effluent from

industrial and sewage are not treated, waste oil from vehicle workshops, waste from restaurants and food premises. Many previous researchers have done studies on the Galing river, that include bioremediation technique to study its potential in degrading pesticide (Ismail, 2008). Galing River water when treated with Granular Activated Carbon (GAC) and Powdered Activated Carbon (PAC) had shown decrement in iron, zinc, and chromium cuprum concentration. According to Malaysia Standard, the water is safe to be used for drinking (Pohan, 2010). From this study, it is shown that the effectiveness of oil degradation is increased by increasing in temperature and the optimum temperature in this study 30 ºC was at low oil concentration (Bahrudin, 2008). Saher et al. (2012), had also reported about a study which mainly proposes a plan and highlights how important to make a sustainable use of Pahang River Basin natural resources in order to ensure the water requirements for its water supplying functions. Some of the methods to solve the problem and the main issue in Galing River today are presented below.

Table 2.2: The Population Surrounding Galing River at 2010

Study Area	Malaysian Citizens					Non-Malaysian Citizens	Total
	Bumiputera		Chinese	Indians	Others		
	Malay	Other Bumiputera					
Area 1	1,358	2	719	389	8	129	2,605
Area 2	2,091	78	1,379	287	5	43	3,883
Area 3	919	32	2,577	355	10	52	3,945
Area 4	1,136	76	4,846	680	31	243	7,012
Area 5	854	9	1,376	285	12	42	2,578
Area 6	567	50	2,926	231	18	145	3,937
Area 7	2,747	84	1,575	229	11	390	5,036
Area 8	2,223	76	4,644	273	32	316	7,564
Area 9	6,293	71	5,850	636	74	341	13,265

2.5 WATER ISSUES AND THE IMPORTANCE OF WATER IN OUR LIVES

Water is one of the important needs in human living. The sources couses of surface water and groundwater. According to Food and Agriculture Organization (FAO), surface water is readily available throughout the year. It is abstracted mainly for Irrigation and domestic uses (Turner et al., 2004). The groundwater potential is limited

to some pockets of the coastal region and is generally exploited by rural people to supplement their piped water supply. Surface water represents 97 percent of the total water use, while groundwater represents 3 percent (Pavelic et al., 2012).

As Malaysia is fast becoming an industrial country, many of the rivers have become polluted due to the many wastes that have been poured out into the rivers (Daneshmand et al., 2011). There are many ethnic aboriginal groups that still exist in Malaysia and the people depend on the rivers and streams to survive. They depend on the river for food, water supply for drinking bathing and for their crops. The river happens to be the main center of their livelihood and without the rivers the whole tribes cannot survive as their ancestors had done generations before them, all of them are dependent on the river. It is very important to make sure that this matter can be overcomes in order to help them save their living resources (Kodori, 2010).

The rapid rate of economic development in Malaysia has caused serious environmental problems in the country, the more prominent being industrial pollution and land development (Snodgrass, 2013). Some of the pollution that may occur include air pollution, noise pollution, water pollution and solid. Wastes pollution and toxic and hazard waste. This study focused on the technology that is being applied in order to treat the water pollution problem.

2.6 WATER QUALITY STANDARD (WQS) TO DETERMINE AND IDENTIFICATION CONDITION CLASS RIVER WATER

2.6.1 Global Environmental Protection

Environmental Protection Administration (EPA) had stated that water monitoring is important to arouse public's concern for protection of water environmental and ensure safe use and water accessibility (Daniel, 2011). Apart from this, this monitoring practice helps in ensuring water quality and assessing the effectiveness of pollution remediation. Thus, a standard of water quality is important for public in order to ensure the water system is protected. Currently, an assessment based of river water quality has been done by EPA (USEPA, 1998). This assessment is known as river Pollution Index (RPI). This index is calculated by using a few parameters such

as dissolved oxygen, biochemical oxygen demand, suspended solid, ammonia nitrogen and another parameter, based on Water Quality Index (WQI) standard.

2.6.2 Malaysia on Water Quality Index system

In Malaysia, the Department of Environment (DOE) had developed a Water Quality Index system (WQI) to analyse trends in water quality of rivers in this country based on seventeen parameters which are known as Dissolved Oxygen, Biochemical Oxygen Demand, Chemical Oxygen Demand, Suspended Solids, Ammonia Nitrate were important of the parameter in the water quality (Bao, 2010). Water quality data were used to determine the water quality, status, whether in clean, slightly polluted or polluted category and to classify the rivers in Class I, II, III, IV or V based on Water Quality Index (WQI) and Interim National Water Quality Standards for Malaysia (1NWQS) every year. The river classification is based on the INWQI as given in the Table 2.3, which shows the classification based on INWQS and the parameters involved.

Table 2.3: Interim National Water Quality Standards (INWQS) for Malaysia

Parameters	Units	Classes					
		I	IIA	IIB	III	IV	V
Ammonical Nitrogen	mg/l	0.1	0.3	0.3	0.9	2.7	> 2
BOD	mg/l	1	3	3	6	12	> 12
COD	mg/l	10	25	25	50	100	> 100
DO	mg/l	7	5 - 7	5 - 7	3 - 5	< 3	< 1
pH	-	6.5-8.5	6.5 - 9.5	6 - 9	5 - 9	5 - 9	
Colour	TCU	15	150	150			
Salinity	$^{o}/_{o}$	0.5	1	-	-	-	-
Total Dissolved Solids	mg/l	500	1000	-	-	-	-
Total Suspended Solids	mg/l	25	50	50	150	300	> 300
Temperature	^{o}C	-	Normal+2	-	Normal+2	-	-
Turbidity	NTU	5	50	50	-	-	-
Faecal Coliform*	counts/100ml	10	100	400	5000 (2000)@	5000 (2000)	-
Total Coliform	counts/100ml	100	5000	50000	50000	50000	>50000

*Note: N: No visible floatable materials/debris or No objectionable odour or No objectionable taste; *: Geometric Mean, @: Maximum not to be exceeded Source: (Saffran et al., 2001).

2.7 DEGRADATION OF POLLUTANT WASTE COOKING OIL AND PETROLEUM HYDROCARBON IN WASTEWATER TREATMENT

2.7.1 Physical Process to Degrade Waste Cooking Oil

Many physical processes to degrade wastewater pollutant, waste cooking oil, such as bioreactor, biofilter, treatment, sewage treatment plant, effluent treatment plant. Physical water treatment is primarily concerned with filtration techniques. Filtration is a treatment using instrument to remove solids from liquids (Wang et al., 2011; Saher et al., 2012; and Mat et al., 2011).

2.7.2 Biological Process Degrade Waste Cooking Oil and Petroleum Hydrocarbon

Many microorganisms possess the enzymatic capability to degrade waste cooking oil and petroleum hydrocarbons. Some microorganisms degrade alkanes, others aromatic, and others both paraffinic and aromatic hydrocarbons (Asamudo et al., 2005). Often the normal alkanes in the range C_{10} to C_{26} are viewed as the most readily degraded, but low-molecular-weight aromatic, such as benzene, toluene and xylene, which are among the toxic compounds found in petroleum and WCO, are also very readily biodegraded by many marine microorganisms (Bahrudin, 2008; Ting et al., 2009; and Isola et al., 2008). More complex structures are more resistant to degradation, meaning that fewer microorganisms can degrade those structures and the rates of degradation are lower than biodegradation rates of the simpler hydrocarbon structures found in WCO and petroleum. The greater the complexity of the hydrocarbon structure, i.e., the higher the number of methyl branched substituents or condensed aromatic rings, the slower the rates of degradation (Atlas, 1995; Ketan et al., 2009; and Pritchard et al., 1992).

The biodegradation of waste cooking oil and petroleum in the marine and river environment is carried out largely by diverse bacterial populations, including various *Pseudomonas* species. The hydrocarbon-biodegrading populations are widely distributed in the world's oceans; surveys of marine and river bacteria indicate that

hydrocarbon-degrading microorganisms are ubiquitously distributed in the marine environment. Generally, in pristine environments, the hydrocarbon-degrading bacteria comprise of < 1% of the total bacterial population. These bacteria presumably utilize hydrocarbons that are naturally produced by plants, algae, and other living organisms. They also utilize other substrates, such as carbohydrates and proteins. When an environment is contaminated with petroleum or waste cooking oil, the proportion of hydrocarbon-degrading microorganisms increases rapidly. In particular, in marine and river environments contaminated with hydrocarbons, there is an increase in the proportion of bacterial populations with plasmids containing genes for hydrocarbon utilization. The proportion of hydrocarbon-degrading bacterial populations in hydrocarbon-contaminated marine and river environments often exceed 10% of the total bacterial population (Atlas, 1995; Sugai et al., 1997; and Chowdhuri et al., 2011).

2.7.3 Chemical Process to Degrade Waste Cooking Oil and Petroleum Hydrocarbon

The major metabolic pathways for hydrocarbon biodegradation are well known. The initial steps in the biodegradation of hydrocarbons and waste cooking oil are by bacteria and fungi which involves the oxidation of the substrate by oxygenates, for which molecular oxygen is required. Alkanes are subsequently converted to carboxylic acids that are further biodegraded via ß-oxidation (the central metabolic pathway for the utilization of fatty acids from lipids, which results in formation of acetate which enters the tricarboxylic acid cycle). Waste cooking oil and Aromatic hydrocarbon rings generally are hydroxylases to form dials; the rings are then cleaved with the formation of cuticles which are subsequently degraded to intermediates of the tricarboxylic acid cycle. Interestingly, fungi and bacteria form intermediates with differing stereo chemistries, fungi like mammalian enzyme systems form *trans*-dials, whereas bacteria almost always form *is*-dials (many *trans*-dials are potent carcinogens whereas *this*-dials are not biologically active). Since bacteria are the dominant hydrocarbon degraders in the marine environment, the biodegradation of aromatic hydrocarbons results in detoxification and does not produce potential carcinogens. The complete degradation (mineralization) of waste cooking oil and hydrocarbons produces the non-toxic end products carbon dioxide and water, as well as cell biomass (largely protein) which can

be safely assimilated into the food web (Atlas, 1995; Sugai et al., 1997; Chowdhuri et al., 2011; Bengtsson et al., 1981; Mountouris et al., 2002; and Reid and Spencer, 2009).

2.8 BIODEGRADATION OF WASTEWATER TREATMENT

A common problem of wastewater treatment is the ability to control high BOD/COD (Biological Oxygen Demand) levels in a system. The first thought is that there is not enough oxygen in the system to do its job. This is usually based on suspicions that the mechanical processes that generate air and oxygen - in relation to the volume capacity of the system, are not functioning well enough or are inadequate. Traditional methods trend to either increase the oxygen levels (dissolved oxygen) and or increase the retention capacity. This is typically the engineering solution and does not always solve the problem (Jacobson et al., 2008).

In anaerobic conditions, failures are often due to the incorrect levels of carbon, nitrogen and phosphorous. Liebig's Law states that the productivity of any complex system dependent on numerous essential inputs, is limited by the single variable in least supply (Sojka et al., 2003; and Watanapokasin et al., 2008).

In this case, the anaerobic microbes required to perform the degrading function will die if not provided with the correct balance of nutrients. As they oxidize they produce acid, which disrupts the total balance of the microbial populations. Due to the competitive nature of microbes, the non-beneficial bacteria will dominate producing an excessive level of pathogens and toxins. This will lead to "stalling" or gridlock and high BOD's with foul odours.

The indigenous microbial species found in a typical wastewater systems are often overwhelmed by system upsets. Harsh chemicals, disinfectants and chlorination will have a detrimental effect on microbial populations, making the systems less efficient. Sludge buildups occur, tank deposits, backups, floating grease pads, fouled drain fields are just some of the problems encountered when microbial populations are not maintained at optimum levels (USEPA, 2012; Limited, 2012; and Sojka et al., 2003).

With a better understanding of the inhibitory factors which cause the desired microbial populations to diminish, we address the cause of the problem, and only then re-populate the system with the specific microbes (augmentation) in the correct numbers, inoculating it with high CFU counts (colony forming units).

2.9 BIODEGRADATION OF ORGANIC POLLUTANT USING LIPOLYTIC BACTERIA

With the rapid development of the city economy, urbanization has caused many problems of city Eco environment, especially the deterioration of water quality. In recent years, there has been increasing interest in developing in situ techniques for remediation using lipolytic bacteria of contaminated river. As an approach to pollution control that uses microorganisms to degrade environmental contaminants into less toxic forms, bioremediation is becoming more and more popular and publicly acceptable due to its low-cost and environmental safety (Ruyan et al., 2003; Tao et al., 2005; and Vaalgamaa and Conley, 2008).

The immersed packing director is simply a bed of organic material (medium), which is the use of biological contact oxidation technology in wastewater. It is a mixed technology of biological membrane technology, Sieve retention technique, and ecological engineering. At its core is the medium with a higher superficial area that can provide a lactation area for the living of micro-flora, protozoa, and plank tonic organism. It has characteristics of efficiency, stability treatment, continuous improvement, and without extra energy consumption, which make it widely to be used since the end of last century in treating domestic sewage, and industrial wastewater and so on (Yongming et al., 2009; Ketan et al., 2009; and Bragg et al., 1994).

2.10 POTENTIAL OF LIPOLYTIC BACTERIA DEGRADED WASTE COOKING OIL IN THE RIVER WATER

The lipolytic of bacteria in the river water is potential to degrade water pollution, the previous study had reported as shown in Table 2.4. Christian et al. (2008) has reported, that lipolytic bacteria were isolated from the Ottawa River, during the winter.

A total of 434 isolates, mainly from the downstream station, were tentatively classified. The major groups were *Pseudomonas*, *Acinetobacter-Moraxella*, and *Aeromonas*. Though the total number of lipolytic bacteria was fairly constant throughout the winter. Isola et al. (2008) had isolated twelve of bacterial species from the waterr of a crude oil and waste cooking oil polluted river. The ability or inability to grow in the presence of 0.5% (v/v) of diesel oil and potential to degrade the diesel oil was investigated. The four bacteria i.e. *Alcaligenes paradoxus*, *Aeromonas* sp., *B. licheniformis*, and *P. fluorescens* had shown potential to degrade the waste cooking oil and diesel oil.

However, Ting et al. (2009) had reported the degradation efficacy of isolate *P. lundensis* UTAR FPE2 on crude diesel showed rapid degradation in the first three days, with a mean of 69% degradation efficacy. However, from day 4 onwards, mean percentage of biodegradation efficacy decreased to less than 50%. Nevertheless, the increase in diesel biodegradation during the first three days benefited the isolate as more viable cells were recovered. Presence of diesel had also sustained growth of bacterial isolate throughout the 7 days, resulting in higher mean of viable cell count of 6.67 log10 cfu/mL, compared to treatments without diesel which recorded 6.56 log10 cfu/mL. At the end of 7 days, viable cell count for treatment with diesel was significantly higher with 7.2 log10 cfu/mL compared to 5.88 log10 cfu/mL recovered from treatment without diesel. While, *Pseudomonas sp*. Degraded 67.57 % of the oil and *Micrococcus sp*. With 52.95 %. But the mixture of *Micrococcus sp.* And *Pseudomonas sp*. were found to have great potential to degrade diesel engine oil, i.e. 89.98 % (Nikhil et al., 2013).

Table 2.4: Isolated lipolytic bacteria to degrade water pollutant

Bacteria	Source	Amount Concentration		Incubation	Temperature	Time	References
		Bacteria	Pollutant				
Pseudomonas lundensis UTAR FPE2	fuel (diesel) tank of a lorry	8 $\log^{10}$ cfu/mL	69%	48 h	27 ± 2 ˚C	3 days	Ting et al., 2009
Pseudomonas putida and P.mallei and *Enterobacter cloacae*	soil samples	-	-				Sandhu et al., 2009
Alcaligenes paradoxus, Aeromonas sp., Bacillus licheniformis, Pseudomonas fluorescens	Water of crude palm oil polluted river and waste cooking oil	-	0.5% (v/v)	48 hours	37°C	5 days	Isola et al., 2008
Micrococcus sp. and *Pseudomonas sp*	garage soil (petroleum contaminated soil), waste cooking oil	*Pseudomonas sp*. was 9.55 x 10-4gm/hr while the mixture of both bacterial isolates showed highest rate of degradation of diesel engine oil i.e. 1.27 x 10-3gm/hr	7.48 x 10^{-4}gm/hr	1 week	37°C	5 days	Nikhil et al., 2013

2.11 ENVIRONMENTAL POLLUTION TREATMENT

It is important to define bioremediation within the context of degradation, a naturally occurring process. Biodegradation using lipolytic bacteria is a large component of oil weathering and is a natural process whereby bacteria or other microorganisms alter and break down organic molecules into other substances, eventually producing fatty acids and carbon dioxide (Hoff, 1993; EPA, 1990; and Allan et al., 2006). Bioremediation is the acceleration of this process through the addition of exogenous microbial populations, through the stimulation of indigenous populations or through manipulation of the contaminated media using techniques such as aeration or temperature control (Swannell et al., 1996; Hoff, 1993; and Atlas, 1995).

2.11.1 Bioremediation Waste Cooking Oil Using Lipolytic Bacteria

The use of microorganisms, fungi or bacteria, is to decompose pollutants into simpler compounds called bioremediation. The process of microbes breaking different substances down into water, carbon dioxide, and other compounds are called degradation (Asamudo et al., 2005; Bahrudin, 2008; and Diaz, 2004). The prime goal of bioremediation using lipolytic bacteria is to create an optimal environment for the microbes to degrade pollutants (Perfumo et al., 2007). There are two types of bioremediation. The first type, bio augmentation, is seeding the water which means adding the microbes to the area. The second is bio stimulation, which is adding fertilizer and nutrients to water to increase the current microbes' degrading capabilities (Zhu et al., 2001; and Hawkins, 2001). The use of bioremediation is often as a secondary treatment tool and is used once other methods have been used to cleanup part of the spill (Venosa, 2006). Bioremediation using lipolytic bacteria is a cost effective alternative but is a very slow process, sometimes taking weeks to months for results. An advantage of bioremediation is that the microbes are able to completely destroy the toxic hydrocarbon compounds and does not just transfer them to another area.

The effectiveness of bioremediation using lipolytic bacteria is difficult to determine. Recreating field conditions in a laboratory is almost impossible to do. That is why field studies, such as at the Exxon Valdez oil spill, are performed whenever

possible. Well-designed field studies can determine if oil is disappearing faster with bioremediation than without and if biodegradation is the main reason for the disappearance (Venosa, 2006).

2.11.2 Bio Augmentation Using Lipolytic Bacteria

Bio augmentation is the addition of Table 2.5 population to degrade oil and other hydrocarbons. This is usually not necessary, since these microbes are present in nearly every location. Microbes lipolytic may need to be added if there are certain contaminants that the resident microbe population is unable to degrade (Venosa, 2006; and Hoff, 1993).

In order to utilize microbes, it is first essential to find which types of microbes are capable of degrading oil and determine their nutrient and environmental requirements. There are 70 genera of microbes that are known to degrade hydrocarbons. Table 2.5 shows some of the more common bacteria and fungi that are capable of degrading hydrocarbons. Usually, only one percent of the natural microbial population is oil degrades. In polluted environments, this amount can rise to more than ten percent of the microbial population (Gordon, 1994; and Swannell et al., 1996).

The degradation of oil will only occur if the other requirements are met, such as available nutrients and the proper environment temperature. The highest concentration of microbes that can survive in most environments is affected by protozoans (who eat microbes), the surface area of the oil spill, and the effect of waves on the area. Added microbes also have a hard time competing with indigenous populations; therefore, bio augmentation is rarely the chosen method. Bio augmentation has never shown long term beneficial results (Gordon, 1994; Hoff, 1993; and Venosa, 2006).

Bio augmentation, when used, usually shows positive results in the destruction of different hydrocarbons. However, it is difficult to determine if the destruction is due to the additional microorganisms or some other entity. Some researchers have worked on developing a microbe lipolytic that can destroy multiple types of hydrocarbons and other oil compounds. These genetically engineered organisms would be able to degrade

all the necessary toxic compounds. Actual use of these organisms is questionable because of the public's negative response to letting a genetically engineered organism free into the environment without knowing the possible consequences. (Xiang et al., 2013).

Table 2.5: Many Microbes Used in Bioremediation (Gordon, 1994)

Bacteria	***Fungi***
Achromobbacter	Allesheria
Acinetobacter	Aspergillus
Actinomyces	Aurebasidium
Aeromonas	Botrytis
Alcaligenes	Candida
Arthrobacter	Cephaiosporium
Bacillus	Cladosporium
Beneckea	Cunninghamella
Brevebacterium	Debaromyces
Coryneforms	Fusarium
Erwinia	Gonytrichum
Flavobacterium	Hansenula
Lactobacillus	Helminthosporium
Leucothrix	Mucor
Moraxela	Oidiodendrum
Nocardia	Paecylomyces
Peptococcus	Phiolophora
Psedomonas	Penicillium
Sarcina	Rhodosporidium
Spherotilus	Rhodotonula
Spirilium	Saccharomyces
Streptomyces	Saccharomycopisis
Vibrio	Scopulariopsis
Xanthomyces	Sporobolomyces
	Torulopsis
	Trichoderma
	Trichosporon

2.12 MODIFICATION OF BACTERIAL CULTURE TO CONTAMINATED SITES USING BIOREMEDIATION METHOD

There are several different bioremediation techniques. The underlying idea is to accelerate the rates of *natural* waste cooking oil biodegradation by overcoming the rate-limiting factors. Several techniques can lead to the results striven for. Indigenous

populations of microbial bacteria can be stimulated through the addition of nutrients or other materials. Exogenous microbial populations can be introduced in the contaminated environment. The addition of extra bacteria is known as bio augmentation. If necessary, genetically altered bacteria can be used. Once the bacteria are chosen, the engineer must carefully meet their nutritional needs by choosing the correct mix of fertilizer (Irwin, 1996; and Shakeri and Moore, 2010). Furthermore, the contaminated media can be manipulated by, for example, aeration or temperature control. Two of these concepts shall be observed in more detail: seeding with microbial cultures and environmental modification.

2.12.1 Seeding with Microbial Cultures to Degrade Waste Cooking Oil and Hydrocarbon

One approach often considered for the bioremediation of petroleum and waste cooking oil pollutants after an oil spill is the addition of microorganisms (seeding) that are able to degrade hydrocarbons. Most microorganisms considered for seeding are obtained from enrichment cultures from previously contaminated sites. However, because waste cooking oil and hydrocarbon-degrading bacteria and fungi are widely distributed in marine and river, freshwater and soil habitats, adding seed cultures has proven less promising for treating oil spills than adding fertilizers and ensuring adequate aeration. Most tests have indicated that seed cultures are likely to be of little benefit over the naturally occurring microorganisms at a contaminated site for the biodegradation of the bulk of petroleum and waste cooking oil contaminants (Ketan et al., 2009; and Atlas, 1995).

2.12.2 Environmental Modification to Degrade Waste Cooking Oil and Hydrocarbon

Waste cooking oil and hydrocarbon biodegradation in the river and marine environments is often limited by abiotic environmental factors such as molecular oxygen, phosphate and nitrogen (ammonium, nitrate and organic nitrogen) concentrations. Rates of waste cooking oil and petroleum biodegradation are negligible in anaerobic sediments because molecular oxygen is required by most microorganisms

for the initial step in waste cooking oil and hydrocarbon metabolism. Oxygen, however, is not limited in well aerated (high energy) marine environments (Ketan et al., 2009; Atlas, 1995; and Swannell et al., 1996). Usually, marine waters have very low concentrations of nitrogen, phosphorus and various mineral nutrients that are needed for the incorporation into cellular biomass, and the availability of these within the area of hydrocarbon degradation is critical (Jio et al., 2011; and Nerurkar, 2007).

2.13 TECHNIQUES FOR ISOLATION OF PURE CULTURE

To understand the differences between the different types of organisms, it is necessary to isolate them into individual colonies. In order to obtain pure colonies, it is necessary firstly to remove all living material from the growth media. The streak plate technique can be used to obtain pure cultures of bacteria from a sample of mixed organisms. Inoculation consists of removing the bacteria from a starter plate and transferring it to a sterile, nutrient agar plate. This spreading of bacteria with a loop is called streaking and results in single colonies of bacteria appearing which can then be identified by different methods (Christopher and Bruno, 2003).

Once the bacterial cultures have grown, a small sample can be taken for Gram-staining. The Gram-stain is the most important and universally used stained technique in the bacteriology laboratory. It is used to distinguish between Gram-positive and Gram-negative bacteria, which have a distinct and consistent difference in their cell walls (Bergey et al., 1994; and Alfred, 2005). Stain or dye reacts chemically with the bacterial cell but not with the background, allowing the bacteria to be distinguished. The shapes of the cell become very obvious, consisting of cocci (spherical), bacillus (rod-like), or spiral (curved), with some indeterminate types (Liu Meng, 2011).

The identification of bacteria is a careful and systematic process that uses many different techniques to narrow down the types of bacteria that are present in an unknown bacterial culture. Traditional methods of bacterial identification rely on phenotypic identification of the causative organism using Gram staining, culture and biochemical methods. The techniques used at the earliest stages are relatively simple. Biochemical tests identify the main biologically important chemical compounds. However, these

methods of bacterial identification suffer from two major drawbacks. First, they can be used only for organisms that can be cultivated in *vitro*. Second, some strains exhibit unique biochemical characteristics that do not fit into patterns that have been used as a characteristic of any known genus and species (Zaid et al., 2001; and Liu Meng, 2011).

In the recent 25 years, molecular biology has developed rapidly and it is now possible to sequence the proteins from different bacterial species, make large database of the sequences, and use them as very powerful identification tools. Similar database has been developed for bacterial DNA and bacterial RNA, particularly the RNA that forms the structural components of bacterial ribosomes. Agarose gel electrophoresis is a method used in molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments by charge (Kryndushkin, 2003).

The polymerase chain reaction (PCR) represents a major advance in terms of the speed, sensitivity and specificity of diagnostic methods, and has been increasingly used to identify several bacterial species from food and clinical samples. Another advantage is that PCR is not dependent on the utilization of a substrate or the expression of antigens, thereby circumventing the phenotypic variations in biochemical patterns and lack of detectable antigens (Hoorfar et al., 2003; and Kevin and Xuan, 2001).

2.14 BACTERIAL IDENTIFICATION METHODS

Identification of unknown microorganisms at the species level with conventional biochemical tests, such as sodium chloride tolerance, citrate utilization, and tobramycin susceptibility, takes days to weeks and can be laborious (Yakrus et al., 2001; and Jaak et al., 1999). For species-level identification of isolates, nucleic acid amplification tests such as polymerase chain reaction (PCR) restriction analysis or DNA sequencing are used widely nowadays (Heller et al., 2008).

2.14.1 Conventional Method

The traditional method to identify unknown bacteria is the biochemical test. In the course of this method, a single colony is used to streak a nutrient agar plate to

determine if the unknown bacteria are Gram positive or Gram negative. The Gram-staining dye will enter the Gram positive bacteria and inhibit growth, but Gram negative bacteria are protected by their enhanced cell wall (Skovbjerg, 2010; and Kranz et al., 2006).

Secondly, bacteria are unable to carry out phagocytosis for acquiring food materials and therefore they excrete exonyms that split larger molecules into smaller units. A variety of proteases degrade protein molecules, such as casein and gelatine, into amino acids, and fats or triglycerides are split into fatty acids and glycerol by various lipases. Bacteria also hydrolyse small molecules, producing signature compounds that can be used in identifying them. Tryptophan is split biochemical test specifically for the indole ring. Likewise, some bacteria degrade urea producing carbon dioxide and ammonia, which causes a colour change in pH indicators owing to the alkaline conditions produced by ammonia (Christine et al., 2008 and Pan et al., 2004).

Apart from the above biochemical tests, there are several additional physiological tests used in unknown bacterial identification. They include tests for hydrogen sulphide production, citrate utilization, phenylalanine deamidisation, and litmus milk reactions (Alfred, 2005; and Lee, 2006).

2.14.2 Identification of Molecular Biology Method

In the past decade, molecular techniques have proven beneficial in overcoming some limitations of traditional phenotypic procedures for the detection and characterization of bacterial phenotypes. Several non-culture based methods have emerged in the past 15 years. The polymerase chain reaction (PCR) and microarrays are currently the most commonly employed molecular techniques. PCR is highly sensitive and allows identification of bacteria at species level. Microarray based bacterial identification relies on the hybridization of pre-amplified bacterial DNA sequences to arrayed species-specific oligonucleotides. Each probe is tagged with a different coloured dye which fluoresces upon hybridization.

Primers targeting the internal transcribed spacer of the unknown bacteria, including a unique reverse primer forward primer, DNA extraction and multiplex, PCR with SYBR Green detection dye on PCR instrument are performed as described by (Kevin and Xuan, 2001; and Lior and Johnson, 1991). Amplified products in the melting curve plot are analysed for their intensity and specificity. Peaks with intensities of greater than 30 you are considered positive (Pinto et al., 2005; and Widjojoatmodjo et al., 1994).

2.15 BERGEY'S MANUAL

Bergey's manual is a manual that use to identify the microbe as to their genus and species. Below are some examples of the identification flow chart of Bergey's Manual Gram Stain & Morphological Flowchart show in Figure 2.4 and Figure 2.5

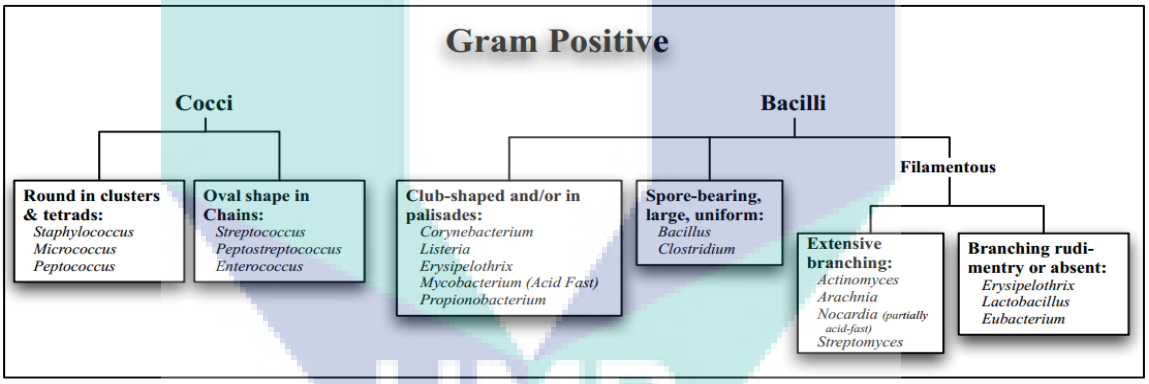


Figure 2.4: Differentiation via Gram stains and cell morphology (Gram Positive).

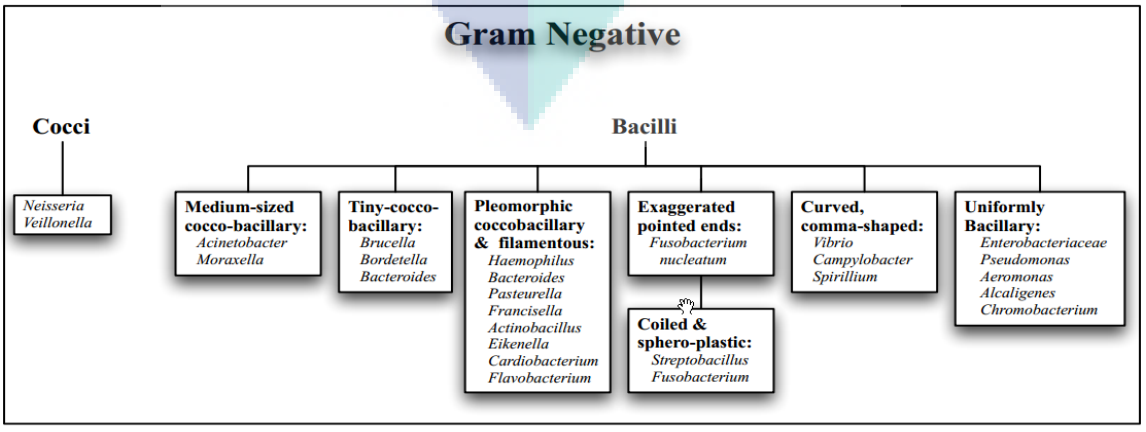


Figure 2.5: Differentiation via Gram Stains and Cell Morphology (Gram Negative)

This study provides a wide range of literature related about the varying process of waste pollutant in the Water River, also waste of industrial, household, hotel, urban and workshops. There are several ways to control the environmental impact of wastewater in the river using bioremediation, bioreactor, biofilter, waste water treatments, biotechnology, chemical processes, biological processes, physical processes, biomembrane, treatment plant, effluent treatment plant, river management and other. Methods to repair streams contaminated of waste, each of which has advantages and disadvantages in improving these conditions, in terms of cost efficiency, availability of materials, time to parse and easy to apply.

The study of literature has concluded that using biotechnology methods are low cost, efficient, and easy to apply. The material is available on contaminated site and we can take advantage of the bacteria that have the capability to degrade the waste in improving the condition of the polluted river. Some variation of method and treatment was studied by the previous researchers. However, the use of bacterial method of isolation from contaminated locations for treatment of water pollution is more interesting to further research. The identification of bacteria using chemical tests, then purification and special treatment of bacteria for identifying ability of bacteria to degrade wastewater for specific research methods is discussed in chapter 3.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 MATERIALS

The materials for identification and culture of bacteria using nutrient agar (NA), Luria broth media (LB), nutrient broth (NB), waste cooking oil (WCO), $CaCO_3$, NH_4NO_3, $Na2HPO_4.7H_2O$, $KH_2PO_4.MNCL_2.7H_2O$, $MgSO_4.7H_2O$, yeast extract, MacConkey agar, Rhodamine B (olive oil, NaCl, agar, nutrient broth), peptone, phenol red and Gram-staining reagents were supplied by Merck, Malaysia Division. G-spin Genomic DNA extraction kit (for bacteria) and MEGA quick-spin PCR & Agarose Gel DNA Extraction System were purchased from Intron Biotechnology, Inc. Agarose power was purchased from Biosyntech Sdn. Bhd. SYBR safe DNA gel stain and 1 Kb plus DNA ladder was purchased from Invitrogen, USA. The TAE - buffer was purchased from DKSH Technology Sdn. Bhd. *Taq* DNA polymerase, $MgCl_2$ and 10 X Buffer were purchased from Invitrogen, USA.

3.2 LOCATION AND COLLECTING OF WATER SAMPLES

Water samples collected from Galing river (Kuantan), sampling station is near Kilang Jaya Gading, (M1) Street Industrial Semambu 4 (N. 03° 50.866’ E. 103° 19.976’), Setali area, (M2) Drain Lorong Seri Setali 60 (N. 03° 50.417’ and E. 103° 19.499”), (M3) Drain Street Galing 33 (N. 03° 50.022’ and E. 103° 19.863’), and (M4) Downstream Vistana Hotel (N. 03° 48.955’ and E. 103° 20.279’). Water samples were collected using sterile glass bottles with Depth Integrated Sample method. The Galing River water was preserved at low temperature before the commencement of the experiment (Table 3.1 and Figure 3.1).

The sampling locations were selected based on initial observations in the field, based on the length of the Galing River and regional division in the area of the Galing river, namely residential areas, offices and public area, industrial and agricultural areas. Galing river is divided into 4 upstream areas, middle upstream, middle downstream and downstream, is also seen by naked eye good water conditions, water color, odour, and levels of contamination by various wastes. An analysis of water quality of the Galing river was done using water quality index standard (WQIS), as a basis for determining the location of sampling points isolation of bacteria that have the ability to degrade the waste.

Table 3.1: Water Sampling Locations from Galing River

No	Locations	Landmark	Latitude (N)	Longitude (E)
1	M1	Street Industrial Semambu 4	03° 50.866’	103° 19.976’
2	M2	Drain Lorong Seri Setali 60	03° 50.417”	103° 19.499”
3	M3	Drain Street Galing 33	03° 50.022’	103° 19.863’
4	M4	Downstream Vistana Hotel	03° 48.955’	103° 20.279’

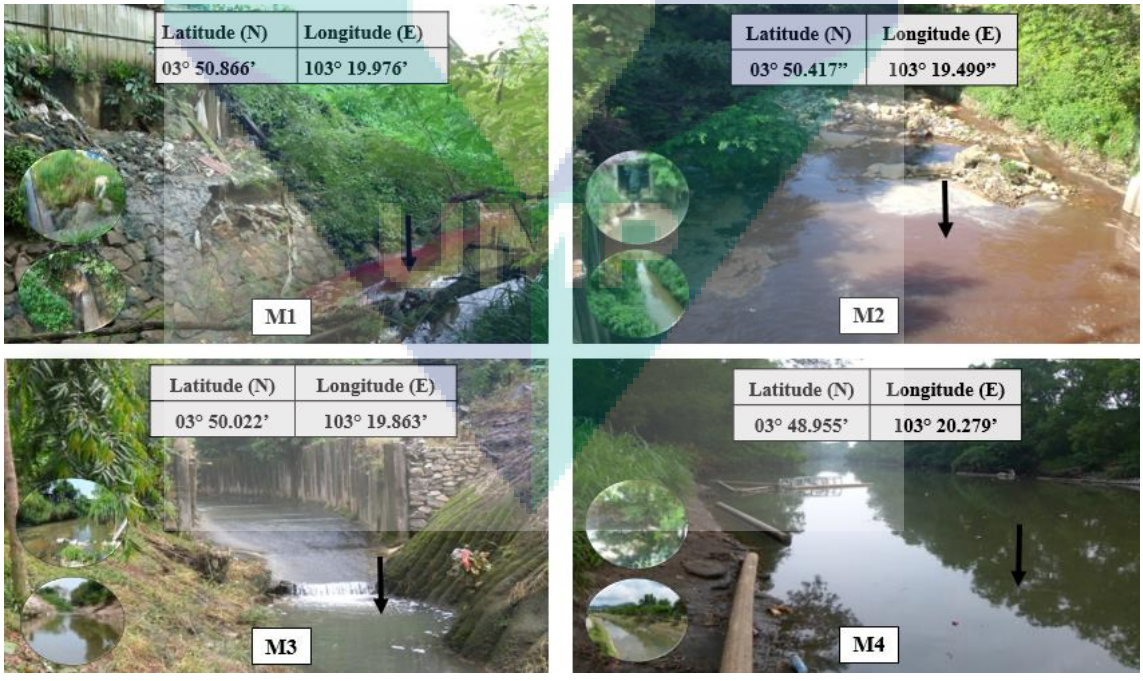


Figure 3.1: Location sampling points in Galing River (M1) Street Industrial Semambu 4, (M2) Drain Lorong Seri Setali 60, (M3) Drain Street Galing 33, (M4) Downstream Vistana Hotel.

3.3 QUALITY ANALYSIS OF GALING RIVER WATER

The location of the data collection and sampling were based on the land use in the Section 3.2. Water and sludge samples were collected using sterile glass bottles with methods *Depth Integrated Sample*. Samples were preserved in the icebox and were sent to the laboratory as soon as possible and kept refrigerated for laboratory testing. All samples were properly labelled with date, time, location and weather condition during time of sampling noted. Table 3.2 shows the parameters sampling and analysis methods which were tested *in situ* and in grab sampling technique.

Table 3.2: Sampling and Analysis Methods

No	Parameter	Sampling Method	Analysis Method
1	pH	In-situ	APHA 4500-H+B
2	Temperature (^{0}C)	In-situ	APHA 2550 B
3	Dissolved Oxygen (DO) (mg/L)	In-situ	APHA 4500 O G
4	BOD (mg/L)	Grab	APHA 5210 B
5	COD (mg/L)	Grab	HACH 8000
6	Ammonical Nitrogen (mg/L)	Grab	APHA 4500 NH3
7	Total Suspended Solid (TSS)	Grab	APHA 2540 D
8	Iron (Fe) (mg/L)	Grab	HACH 8147
9	*E.coli*	Grab	APHA 9222 G
10	Oil and Grease	Grab	USEPA 10056
11	Water Quality Index Standard (WQI)	Interim	NWQI

**American Public Health Association (APHA)*
**United States Environmental Protection Agency (USEPA)*
**HACH method is accepted by USEPA for reporting wastewater analysis*
**Environmental Protection Agency (EPA)*

Water samples were collected through *in-situ* and grab sampling technique and was preserved and analyzed in the lab. Water quality parameters that need to tested *ex-situ* are pH, temperature and dissolved oxygen (DO), while water quality parameters that were tested in the lab are Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Ammonia Nitrogen (NH_3-N), Total Suspended Solids (TSS), Iron (Fe) using DR-2800 spectrophotometer, *E. coli* using *E. coli* Quanty-try, and Water Quality Index Standard (WQI).

The relevant testing standard used is MS ISO/IEC 17025 'General Requirements for The Competence of Testing and Calibration Laboratories' and APHA methods. The tests have been conducted in the Faculty of Science and Technology, University Malaysia Pahang (UMP), Kuantan, Pahang.

3.4 ISOLATION OF BACTERIA FROM GALING RIVER WATER

The sample bacteria were isolated from M4 Vistana Hotel point sampling in Galing River. Point sampling have been based on observation, polluted conditions than the other locations sampling. The preparation media from commercial products, is according to the manual. Normally, to prepare agar plates, requires 24 grams of nutrient agar (NA) in 1L distilled water. The suspension is mixed in an Erlenmeyer flask, then dispensed and sterilized for 20 minutes at 121 °C. Then the agar was poured into a petri plate, to make an agar plate nutrient. Agar pours containing 15 ml of media are often used to prepare agar plates (Chowdhury et al. 2011).

3.4.1 Determination Bacteria by Quantifying of Population Growth Colony Forming Unit (CFU/mL) and Optical Density (OD600 nm) in River Water Sample

A Galing river water sample was taken to the laboratory and stored overnight at 4 °C. An amount of 1 ml of each water sample was diluted from 10^{-1}, 10^{-3}, 10^{-8} dilution factor. Then, an amount of 100 µl from each dilution tube was inoculated into a prepared petri dish containing sterile nutrient agar. The plates were inverted and placed in the incubator at 37 °C for 24 hours (Dhall et al. 2012).

Independent colonies that appear to be composed of only single colonies were chosen. An inoculating loop was filmed and cooled, and used to gently transfer the colony by touching, and re-streaking onto a new nutrient agar plate. The plates were then incubated at 37 °C for 24 hours and after accounting of colonies. The development colonies were counted in plates and average number of colonies per three plates were determined. Then the bacterial isolates were kept on nutrient agar at 4 ºC and re-cultured every 4 weeks. The number of total bacteria (CFU) per ml sample was calculated as the following formula:

CFU/ml = (no. Of colonies x dilution factor) / volume of culture plate (Eq. 3.1)

To identify population growth in bacteria, the liquid medium was prepared with 8 grams of nutrient broth (NB) in weight, and then suspended in distilled water. The suspension was mixed thoroughly in an Erlenmeyer flask. Then it was dispensed and sterilized for 20 minutes at 121 °C. Inoculums were prepared in nutrient broth by using an inoculating loop from independent colonies from pure culture. Microbial growth was monitored at regular intervals by measuring the optical density (OD) at 600 nm by using several dilutions in various time and day incubation (Bahig et al. 2008).

3.5 SCREENING THE POTENTIAL OF LIPOLYTIC BACTERIA TO BIODEGRADE OIL USING PHENOL RED, RHODAMINE B AND MACCONKEY

Phenol red agar plate was also used to assess the test organisms. Phenol red agar plates were prepared (% w/v): using phenol 0.01 along with 1 % (v/v) olive oil, 0.1 $CaCl_2$, 2 agar and the pH adjusted to 7.3 – 7.4. About 20 ml of the medium was poured into each petri dish and the organism were inoculated 48 hours. Changes in colour of phenol red were used as an indicator of the enzyme activity.

Agar plate containing (% w/v): Rhodamine B 0.001, nutrient broth 0.8 %, NaCl 0.4, agar 1 and olive oil 2 % was prepared in distilled water, adjusted pH of 6.5. The assay incubated at 55 ºC for 18 h and lipolytic activity was identified as an orange halo around colonies under UV light at 350 nm (Kim et al. 2008).

The media were prepared by suspending 27.75 gr MacConkey agar in 500 ml distilled water. The media were heated to boiling with gentle swirling to dissolve completely. The media were sterilized by autoclaving at 121 ºC for 20 minutes at 151bs pressure. Overheating was avoided. The media were cooled to 45-50 ºC and poured into sterile Petri dishes. The surface of the medium was dried when inoculated (Park et al. 2011).

3.6 CULTIVATION AND MORPHOLOGICAL CHARACTERISTICS OF BACTERIAL ISOLATES

After the isolation process from river water samples, a number of bacteria were sub-cultured and their morphology was observed. The identified bacteria were isolated and subjected to the Gram-staining procedure in order to classify them (Bergey's Manual Determinative Bacteriology). The morphology observation and Gram-staining results were analyzed together. The general characteristics of bacterial colonies were then described in terms of shape, margin, elevation, colour to give an accurate description of the colonies.

3.7 ESTIMATION OF BACTERIAL GROWTH POTENTIAL TO DEGRADATION WASTE COOKING OIL (WCO)

Growth in mono and mixed culture isolates on media containing 10% waste cooking oil carried out to determine the best mix of cultures. Tests were conducted on liquid medium in 100 ml serum tubes containing 17.8 ml minimal medium, 2 ml of WCO and 0.2 ml sterile isolates. Fermentation was carried out on a rocking incubator 120 rpm at room temperature (25-30 °C) for 4 days. Observations were made by measuring the bacterial growth turbidity changes at a wavelength of 600 nm with a spectrophotometer, every day (Dhall. et al. 2012; Diez 2010; Edema 2006; *and* Margesin, 2001).

3.8 TESTING THE ABILITY OF BACTERIA TO DEGRADE WCO AS CARBON SOURCE

Testing the ability of isolates to use carbon sources of waste cooking oil performed on solid media. Isolates were grown in Luria Bertani (LB) liquid medium having 6 hours of solid minerals disseminated in the media, given the sterile 2 pieces of paper containing waste cooking oil. Cultures were incubated at 30 ^{o}C for 2-3 days. Observation of the growth of bacterial colonies around the paper containing substrate is done visually. Bacteria were able to grow and meet the assumed positive filter paper circle, whereas if there was a clear area assumed difficulty or unable to use the carbon source (Dhall. et al. 2012; Diez, 2010; *and* Edema, 2006).

3.9 IDENTIFICATION BACTERIAL WITH RAPID ONE TEST

Inoculum preparation Test organisms must be grown in pure culture and examined by Gram stain and oxides prior to use in the system. Test organisms may be removed from a variety of selective and nonselective agar growth media. The following types of media nutrient agar and MacConkey agar, using a cotton swab or inoculating loop, suspended sufficient growth from the agar plate culture in RapID Inoculation Fluid (2 mL) to achieve a visual turbidity equal to a McFarland turbidity standard or equivalent. An agar plate may be inoculated for purity and any additional testing that may be required to use a loophole of the test suspension from the inoculation fluid tube. Incubate the plate for at last 18-24 hours at 35-37 ºC.

Inoculation of RapID ONE panel peels back the lid of the panel over the inoculation port by pulling the tab marked "feel to inoculate" up and to the left. Using a pipette, gently transfer the entire contents of the inoculation fluid tube into the upper right hand corner of the panel. Reseal the inoculation part of the panel by pressing the peel back tab back in place. Incubation of RapID ONE panel incubates inoculated panels at 35-37 ºC in a non CO_2 incubator for 4 hours. For ease of handling, panels may be incubated in the chipboard incubation trays provided with the kit. And then scoring of RapID ONE using interpretation of RapID ONE system test (Adley and Saieb, 2005).

3.10 PCR AND DNA (ANALYSIS OF 16S rDNA GENE SQUENCES AND PHYLOGENETIC ANALYSIS)

3.10.1 Genomic DNA Extraction

The DNA extraction from samples was carried out, firstly, 3 ml of bacterial culture was grown overnight or culture grown to log phase by centrifugation at 8000 rpm for 2 minutes at room temperature. The supernatant must be completely decanted. Resuspension of pellet by adding 100µl Buffer R1 to the pellet and resuspend the cells completely by pipetting up and down. Lysozyme treatment of Gram-negative bacteria, strains was added 10 µl lysozyme (50 mg/mL) into the cell suspension. For Gram-positive bacteria strains, by adding 20 µl lysozyme (50mg/mL) into the cell suspension. Then it was mixed thoroughly and incubated at 37°C for 20 min. The pellet

digested cells by centrifugation at 10.000 rpm for 3 minutes, and then decanted the supernatant completely. The next steps was protein denaturation by resuspend pellet in 180 µl of Buffer R2 and add 20 µl of Proteinase K. It was mixed thoroughly, then incubated at 65°C for 20 min in a shaking water bath or with occasional mixing every 5 minutes. The next step was removal of RNA, if RNA-free DNA was required, was added 20 µl of RNase A (DNase-free, 20mg/mL), was then mixed and incubated at 37°C for 5 minutes. Then homogenize the sample, by adding 2 volumes (~400 µl without RNase A treatment, ~440 µl with RNase A treatment) of Buffer BG and mix thoroughly by inverting tube several times until a homogeneous solution was obtained, followed by incubation for 10 minutes at 65°C. Addition of ethanol was added 200 µl of absolute ethanol, then mixed immediately and thoroughly. The sample was than transferred into a column assembled in a clean collection tube (provided), then centrifuge at 10.000rpm for 1 minutes. For column washing, with 750 µl of wash Buffer and centrifuge at 10.000 rpm for 1 minutes. Discard flow through. The column drying followed by centrifuge the column at 10.000 rpm for 1 min to remove residual ethanol. Then, column was placed into a clean micro centrifuge tube. 50 - 100 µl to was added the preheated Elution Buffer, TE buffer or sterile water was directly onto column membrane and stood for 2 minutes. Centrifuge at 10,000rpm for 1 minute was to elute DNA. DNA was stored at 4°C or -20°C freezer (Pinto et al. 2005).

3.10.2 PCR Amplification with Universal Primers and PCR Product Purification

DNA concentration was determined by using UV-VIS spectrophotometer under 260nm wavelength on samples diluted 50 times by mixing 1µl samples with 49 µl distilled water, and then DNA concentration was calculated as follows:

$$\text{DNA concentration} = OD_{260} \text{ X } 50\text{ng/µl X } 50 \text{ (Dilution Factor)} \qquad \text{(Eq. 3.2)}$$

Published 16S rRNA sequence was available in three *Pseudomonas* species for the use in the design of primers for PCR, these sequences were filed in the international gene banks. Two oligos universal primers were designed for use in PCR. The first forward primer (F: 5’ AGA GTT TGA TCC TGG CTC AG3’) and one reverse primer

(R: TAC GGY TAC CTT GTT ACG ACT T3') for use in initial PCR amplification (Pinto et al. 2005).

The concentration of DNA sample was estimated by measurement of absorbency at 260 nm on a spectrophotometer. Amplification conditions were run for 30 cycles. A typical reaction mixture in each PCR tube for 20µl total volume, containing 1µl of the appropriate dilutions of DNA, 2µl of 10 X PCR reaction buffer, 0.5µl of DNA polymerase, 0.5µl of dNTPs, 1µl of $MgCl_2$, 1µl of each primer. The mixture was brought to 20µl with sterile deionised distilled water. All PCR reactions were run with a negative control (no DNA). PCR involved initial denaturation at 94°C for 1 min, five cycles with a low annealing temperature of 50°C for 5 s, extension at 72°C for 30 s and heated to 94°C for 5 s, and additional 25 cycles of denaturation at 92°C for 2 s, annealing temperature at 55°C for 2 s, extension at 72°C for 30s and final extension at 72°C for 2 min (Gibson, 2006).

The PCR products were then separated on a 1% agarose gel containing 1 mm ethidium bromide for visualization on a Bio-imaging machine. The interesting DNA fragment was cut out with a sharp scalpel after PCR product electrophoresis and was taken care as much as agarose gel as possible. The gel slice was weighed in a 1.5 ml tube and then 3 volumes of BNL buffer were added to 1 volume of gel (300µl per 100mg of agarose gel). The mixture was shaked and incubated at 55°C for 10 minutes or until the gel is completely dissolved. The dissolved gel mixture was transferred to the kit column assembly and centrifuged for 1 minute; the follow-through was discarded after centrifuge. 700µl of washing buffer was added to the column and centrifuged at 13,000 rpm for 1 minute and discarded the flow-through. The column was placed in a clean 1.5 ml micro-centrifuge tube after centrifuge for 1 min at 13,000 rpm to dry the spin membrane. 60µl of the elution buffer was directly applied to the centre of the column without touching the membrane with the pipette tip. The tube was incubated at room temperature for 1 min and centrifuged for 1 min at 13,000rpm. Lastly tubes were stored at minus 20°C (Kevin and Xuan, 2001).

3.10.3 Electrophoresis Visualisation of Extracted DNA and PCR Products

Agarose gel electrophoresis is the easiest and commonest way of separating and analysing DNA. The purpose of the gel were be to look at the DNA, to quantify it or to isolate a particular band. The DNA is visualized in the gel by the addition of ethidium bromide. This binds strongly to DNA by intercalating between the bases and is fluorescent, meaning that it absorbs invisible UV light and transmits the energy as visible orange light. So the detailed steps are as follows: weigh 0.3 gr agarose power, suspended in 30 ml 1X TAE buffer, and thoroughly dissolved in a microwave oven for 2 min. Add 3µl ethidium bromide before pouring the gel when the agarose have cooled to about 55° C. The proper comb was inserted for the particular gel rig. The gel were allowed to cool until it has set. The comb was carefully removed and placed the gel in the gel rig with the wells closest to the cathode (black) end. The gel was covered with 1X TAE running buffer. 3µl of glycerol loading dye was placed onto the waxy side of parafilm for each sample. Keeping samples on ice, 1µl of a sample was added to one of the drops of loading dye. The sample and loading dye was mixed by filling and emptying the pipette a few times, then the mixture was loaded into a well. The rest of the samples were loaded one by one as same as the first one sample. 4µl of 1 Kb ladder was loaded at the left end of the series of samples. The cover was placed on the gel rig and samples were ran towards the anode (red) end at 90V for 40min. The power pack was a turned off and the gel was removed out. Visualization was carried out with U.V. The light and the photograph were taken with a Polaroid Photo documentation camera. Lastly the waste gel was disposed properly (Jaffe et al. 2001).

3.10.4 Sequence Analysis and Blast Analysis

The PCR products were sent to the expert company (1st BASE, USA) to analyse the sequence, and then Blast the sequence with Gene bank.

3.11 BIODEGRADATION OF WCO USING ENRICHMENT BACTERIA ISOLATES FROM GALING RIVER WATER IN BATCH PRODUCTION

Growth studies of sewage bacterial isolates was studied in 250 ml flasks containing 50 ml LB medium supplemented with 0.1 mL concentration of Waste

cooking oil. Flasks were inoculated with 0.5 ml of overnight culture and agitated on a rotary shaker (150 rpm) at 30 ^{o}C. Growth was monitored as a function of biomass by measuring the absorbance at 600 nm using a spectrophotometer (Hitachi, Japan).

Insulation was made in four stages, using a sample of waste cooking oil "WCO" of household waste, as a source of isolates. Basal medium used was "Stone Mineral Salt Solution" (SMSs) consisting of (g/L) 5 $CaCO_3$, 2.5 NH_4NO_3, 1 $Na_2HPO_4.7H_2O$, 0.5 KH_2PO_4; 0.5 $MgSO_4.7H_2O$ and 0.2 $MnCl_2.7H_2O$ dissolved in 1 litre of distilled water Yeast extract as much 0.01 % (w/v) was added to the medium as the SMSs additional nitrogen source. Medium is added to leftover cooking oil 2 % (w/v) as a carbon source. pH was adjusted to be 6.8 to 7 (Karigar et al. 2006).

3.11.1 Determination Growth Bacteria in Batch Production Using Variation Concentrations of Waste Cooking Oil (WCO) from M4 Location Sampling

Determination of growth bacteria in batch production and the variance of concentrations of the WCO (1-4%) was added in batch production for 1-4 days of time degradation with optical density analysis were measured. The incubation condition were 30 ºC and the 2% of isolated. Bacteria was added in stone mineral salt solution to enrich (SMSSe) medium for 4 days in incubation with a speed of 120 rpm. The sample was taken each day for OD measuring purposes, then was analysed using UV spectrophotometry in the 600 nm wavelength.

3.11.2 Degradation of Waste Cooking Oil (WCO) Using Variation Amounts of Bacteria from M4 Locations Sampling in Galing River Water with Gravimetric Analysis

Isolation in batch production is preceded by shaking 2% (w/v) “WCO” in stone mineral salt solution in an enrich (SMSSe) medium in 7 days with a speed of 120 rpm. For isolation purposes, sampling was taken each day, then the isolation was done by dilution method. Samples were taken for as much as 1 ml cultured on the plate so that SMSSe containing “WCO” pour plate method. Each different colony was purified again on the same solid medium. The verifying of amount bacteria (50, 75, 100 µl) and concentration of the WCO (1-4%) was also observed. The bacteria were isolated from M4 point sampling in Galing River. Bacterial isolates was obtained and identified

through observation colony morphology, cell and gram straining (Capuccino and Natalie, 2000).

Bacteria were identified from the sampling location M4 amount a 5 I *Providencia stuartii* species, *Bacillus pimilus, Exiquobacterium sp. Bacillus antracis. Ames.* And *Bacillus antracis. Ames* there were two species, of the 5 types of bacteria. All bacteria will be used to degrade the WCO using gravimetric analysis. Then the testing ability of oil degradation by bacterial isolates was done after isolating activated in a medium that has been supplemented SMSSe "Waste cooking oil" and the residual oil degradation had determined the rate of growth. Degradation products were analyzed by calculating the weight of residual oil degradation (Gravimetric). The percentage of degradation was calculated by the following formula:

$$\text{Percent (\%) Degradation} = \frac{\text{Amount of crude oil degraded}}{\text{Amount of crude oil added}} \text{ X } 100 \qquad \text{(Eq. 3.3)}$$

Amount of crude oil degraded = (Weight of crude oil added in the media) – (Weight of residual crude oil)

Amount of residual crude oil = (Weight of beaker + oil) – (Weight Empty beaker) (Latha and Kalaivani, 2012).

3.12 CHARACTERIZATION OF WASTE COOKING OIL DEGRADED WITH BACTERIA ISOLATES USING FT-IR ANALYSIS

An IR region of 400-4000/cm with 16 scan speeds. The samples were fixed with KBr spectroscopic pure chloroform in the ratio of 1:100 and pellets were fixed in the sample holder and the analysis were carried out (Mohanan et al. 2007).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 STUDY AREA LOCATION SAMPLING GALING RIVER WATER

The object of study, as Galing River which is situated in Kuantan city with the catchment area covers 22.7 Km2 and the length of a river line reaches 7.7 km. The catchment area falls under the jurisdiction of the Majlis Perbandaraan Kuantan (MPK) as show in Figure 4.1

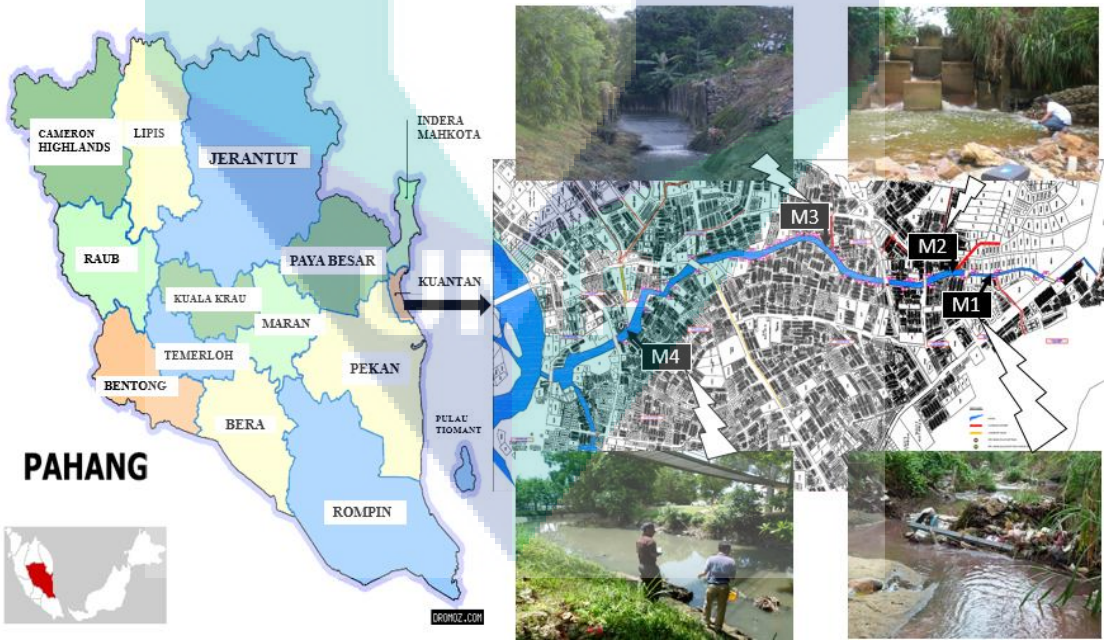


Figure 4.1: Map showing four Locations sampling point in Galing River (M1) Street Industrial Semambu 4, (M2) Drain Lorong Seri Setali 60, (M3) Drain Street Galing 33, (M4) Downstream Vistana Hotel.

According to the data issued by the Department of Environment (DOE) of Malaysia in 2008, there were several sources of water pollution surrounding Galing River which classified into sectors. Based on the data, the composition of water pollution, which has been recorded accounts for 17,633 points in 2008 alone, comprising sewage treatment plants (9,524: 54.01%, including 668 Network Pump Stations), manufacturing industries (6,830: 38.73 %), animal farms (788: 4.48%) and agro-based industries (491: 2.78%).

This study focuses on water pollution caused by the industrial wastewater. These water effluents are emitted by industrial plants which may contain hundreds of thousands of chemicals. Yet only a few of these are responsible for aquatic toxicity (Fahmi et al., 2011; Fakoussa and Hofrichter., 1999; and Field and Samadpour, 2007). The DOE compiles the statistics of industrial water pollution sources from agro based and manufacturing industries, workshop, domestic waste, hotel, restaurants and people activities.

4.2 CHARACTERIZATION OF WATER QUALITY STANDARDS IN THE APPROPRIATE CLASS OF WATER QUALITY INDEX PARAMETERS

Characterization of water quality standards has to be done to see from four locations in the study, if the water content and the quality is good or bad, then we have to know the location of sampling that contains polluted water which will be made in the location sampling of the isolation of bacteria which will then be used as a test on the ability of bacteria in degrading WCO.

Water pollution is emerging as a threat to all humanity, as it determines the health quality of aquatic life for the environmental population surrounding the area of Galing River. Peng et al. (2003) the problem of environmental pollution is generally due to industrialization, urbanization, high density of population and the unplanned introduction of the plant, which are often proved to be hazardous to human health.

The physical, chemical and biological parameters of Galing River, namely, pH, temperature, DO, BOD, COD, TSS, AN and other parameters have shown significant effects on the water quality. The term “water quality” is closely related to watering

pollution. These indicators are represented by physical-chemical parameters from different location of sampling point, among others (M1) Street Industrial Semambu 4, (M2) Drain Lorong Seri Setali 60, (M3) Drain Street Galing 33 and (M4) downstream Vistana Hotel. The parameters are explained in the following sections.

4.2.1 pH

In environmental quality assessment, pH test is a standard of preliminary study. Of the water quality, it is one of very crucial aspects since even small changes in pH (0.3 units or even less) will usually impact of relatively great changes in other water qualities. pH water quality standard measures hydrogen ion concentration within a substance. Value of pH for class I is in the range of 6.5 to 8.5 which indicates a neutral solution, On the other hand, pH indicating value lower than 6.5-8.5 is classified to the acidic solution while solution which pH value higher than 6.5-8.5 indicate alkalinity.

Most of natural water has a pH ranging from 5.0 to 8.5. Water pH from freshly fallen acidic rain might be in the interval of 5.5 to 6.0. When it is exposed to soils and minerals containing weak alkaline materials, the concentration of hydrogen ion decreases (Dassenakis et al., 1998). Thus, water becomes slightly alkaline, with a pH of 8.0-8.5. Waters with an acid value more than 5.0 or with base value more than 8.5 to 9.0 has to be considered with cautious assessment. pH which suddenly changes act as an indicator of warning signals that water quality may adversely be introduced to some contaminants. In water quality analysis, pH of water samples can be determined *in-situ* (on site analysis) or the samples can initially be collected from the site and pH will be determined afterward. Most of aquatic systems have a pH in the mid-range of the pH scale. Therefore, for this analysis, a pH indicator with the scale ranging from 5 to 9 is considered sufficient (He et al., 2007).

Figure 4.2 shows that the sample water collected from sampling M1 has the lowest pH value of 6.61, while that for sampling location M4 indicates the highest value of pH which is 6.95. It is influenced by time and weather measurements in the environment that there isn't difference, the measurement in the morning is not a lot of activity sewage into the river and relatively clean. There is no significantly different pH

in each location sampling since the value of all location sampling is in the range 6.5 < pH value < 9.0. According to the standard INWQS it can be concluded that all the location of sampling points from M1 to M4 are categorized into class I and II. Since water generally becomes more corrosive with decreasing pH, water from the M1 location sampling point is the most corrosive from other locations since its pH value is the lowest among all of the other sampling points.

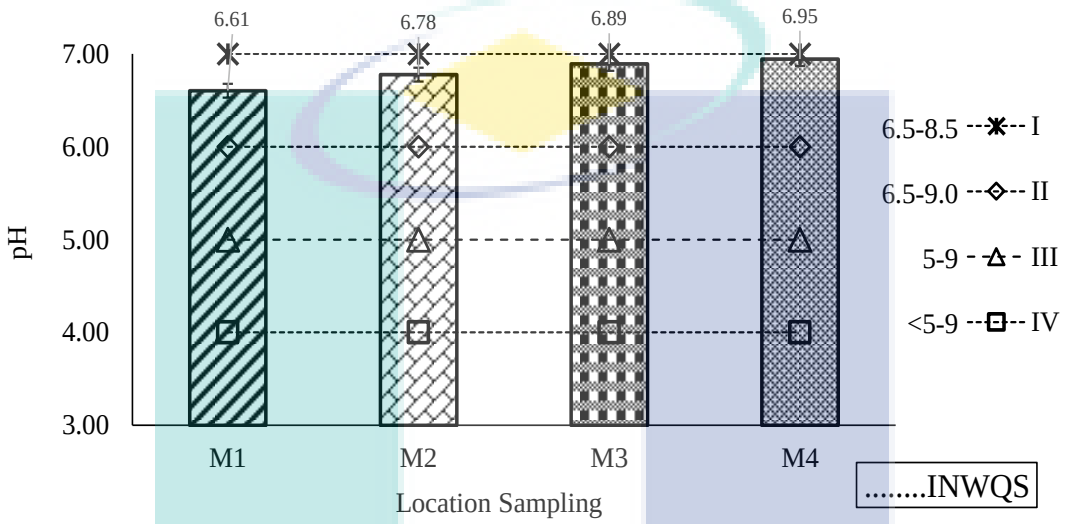


Figure 4.2: The average pH values for 4 sampling points along the Galing River water in Water quality standard

Based on the above explanation, it can be concluded that the condition of the Galing River at M1 and M2 sampling points is categorized into class I whereas those at sampling points of M3 and M4 fall into category of class II with a normal pH 6.5 - 8.5 range of indigo. This result of the study confirms with other findings which stated that this condition is normal and reasonable in the river and considered not too polluted by sewage (Cserháti et al., 2002; Ahmad et al., 2008; and Madrid and Zayas, 2007).

4.2.2 Temperature

The water temperature must be measured *in-situ* and should be sampled at various depths using a recording Celsius thermometer. For streams and rivers, readings were taken at intervals relative to the water depths. Location of temperature reading, edge, middle, inlet, etc. was also considered. From Figure 4.3 it can be shown that the all points sampling (M1 to M4) have similar temperature in the range of 27 to 32 ˚C.

The maximum temperature reading is at 31 ˚C in M4 sampling point, while the minimum temperature is 27.28 ˚C in M1 sampling point. The increase and decrease of water temperature in the river depend greatly on the climatic condition, sampling time, the duration of sun exposure, and also affected by the unique characteristics of water surrounding environment such as turbidity, wind force, vegetation pattern, and humidity (Mahmoud, 2002; Tayel, 2002 and Sabae et al, 2008). The temperature of the water, which relatively increases at M2 sampling point during testing is due to the thermal pollution released to the M4 sampling point from the multiple wastes Vistana Hotel located nearby the location sampling.

From the explanation above, it can be concluded that the Galing River has good and normal thermal condition, which is in the range of 27 - 32 ºC. It is influenced by time and weather in environmental measurements, measurements made on the morning of the relative has not received the scorching sun. Whereas measurements were taken during the day or more will affect the temperature in the environment. This means that this condition is reasonable and normal in sunny weather conditions. Sewage into the river and the river condition itself are shaded with trees, making the surrounding environment is in good condition, according to research carried out in other areas by Berberoglu and Akin, (2009).

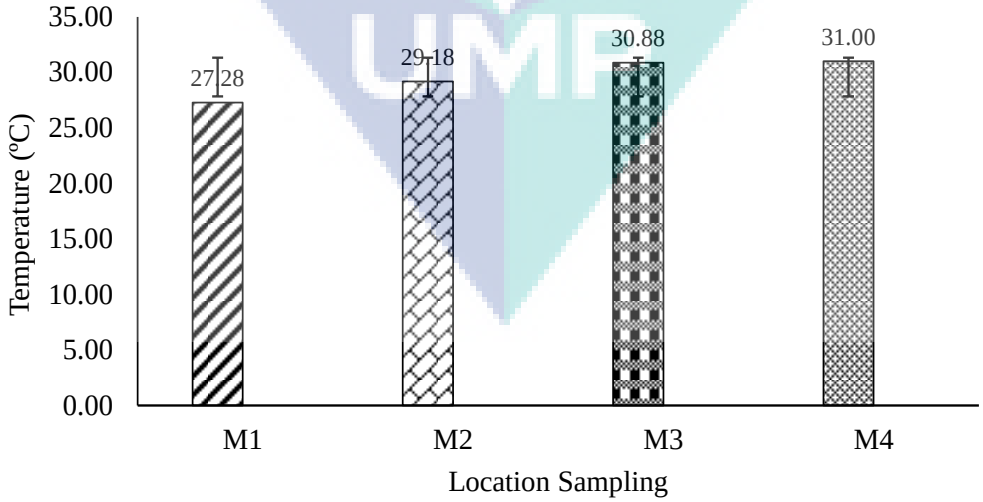


Figure 4.3: The average temperature values for 4 sampling point along of the Galing River water

4.2.3 Dissolved Oxygen (DO)

For aquatic living, such as organisms, dissolved oxygen is very crucial factor. This term refers to the measure of the gaseous oxygen amount (O_2) which is dissolved in an aqueous solution. The lower the concentration of dissolved oxygen, the greater the stress organisms will incur. The process of natural stream purification demands sufficient level of oxygen in order to maintain living for aerobic life. The level of oxygen, which remains less than 1 to 2 mg/l for several hours will cause death to large fish.

Based on Figure 4.4 the observation result indicates that the dissolved oxygen of all locations of sampling point in the range of 2.5 to 3.8 mg/L with minimum level as low as 2.5 mg/L at location sampling point M4 and the maximum level reaches 3.8 mg/L at location sampling point M3. According to the result mentioned, all locations of sampling points are classified as a class III except M4 which is classified into grade IV based on INWQS and DOE index. It indicates that all location sampling points fall into class IV and are suitable for irrigation based on the classification in the DOE water quality index (Bao, 2010; and Bartram and Balance, 1996).

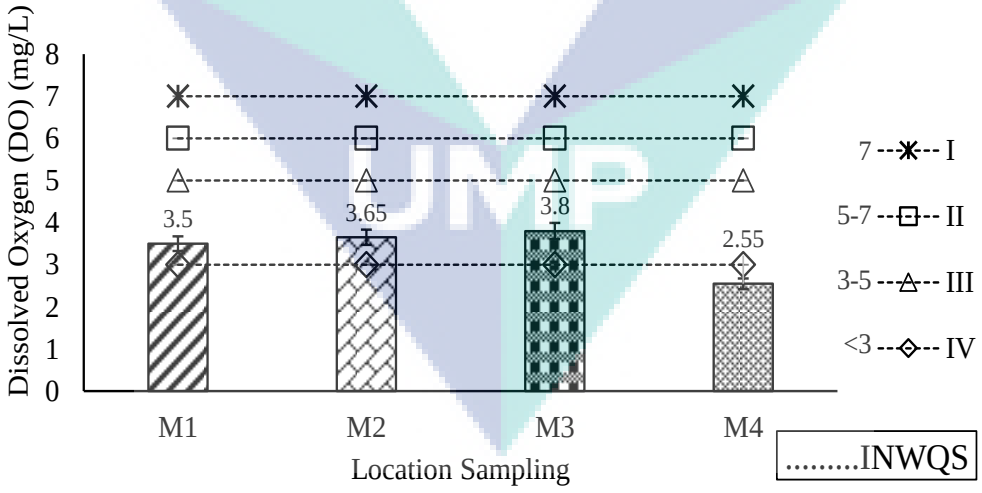


Figure 4.4: The average Dissolved oxygen (DO) (mg/L) values for 4 sampling point along of the Galing River water in Water quality standard

Temperature, pH and Dissolved oxygen (DO) in Figure 4.2, Figure 4.3 and Figure 4.4, in the environment also have an impact on the lives of micro-organisms in the water, one of which is a waste degraded bacteria, which lives in the environment's

ability to rely on the availability of food resources, temperature pH and DO. In advanced research will be discussed the influence of variations in food sources and the number of bacteria in degrading waste cooking oil.

4.2.4 Biological Oxygen Demand (BOD)

Based on Figure 4.5, it is found that M3 sampling point is the highest reading of BOD concentration compared to other sampling points which can reach 34.19 mg/L, followed by M2 location sampling point (30.91 mg/L), then M1 location sampling point (26.14 mg/L). M4 location sampling point is the lowest in term of BOD which is at 12.76 mg/L. Based on the classification of INWQS and DOE water quality index, all the location sampling points fall into class IV, the value of which >12 mg/L The higher BOD concentration recorded at all sampling stations (M1 to M4) might be corresponding to the high organic matter discharged into the river from industrial activities. Relatively high temperature at these locations can enhance the bacteria population. In addition, the main source of the high organic load in M1 sampling point is the sewage from the slaughtering activities which discharges organic matters, such as blood. This finding is in agreement with that reported by (Muhirwa et al. 2010),stating that wastewater from the abattoir slaughtering area increases the organic load in the Mpazi River. Galing river water in not good condition when viewed from INWQS based on the BOD data available.

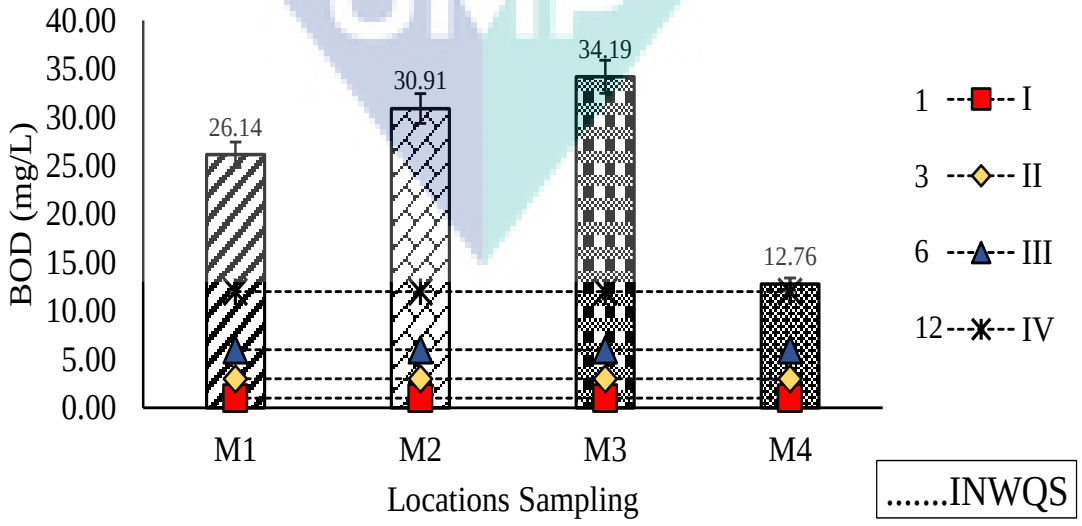


Figure 4.5: The average Biological oxygen demand (BOD) values for 4 sampling point along of the Galing River water in Water quality standard

4.2.5 Chemical Oxygen Demand (COD)

The Chemical Oxygen Demand (COD) is the third most important parameter of River water quality based on WQI. Generally, a value of COD estimates the amount of current organic and inorganic matter. From Figure 4.6, it can be shown that the maximum concentration of COD a concentrations to M4 sampling point, with concentration of COD reaches 200 mg/L. The COD at this level is classified into class V based on INWQS and DOE water quality index in Malaysia. The higher COD concentration at this station may be attributed to the effluents discharged from multiple wastewater of Vistana hotel nearby this area. The high COD concentration of the Nyabugogo river at the level of 355.73 ± 599.57 mg/L is also reported by (Nhapi et al. 2011). The increase of COD level induced from various chemicals such as Iron, Manganese, Sulphates, Phosphates and Nitrogen which are emitted from refineries in these locations. Furthermore, the other locations of sampling point are categorized into class II the COD concentration of which is of value of 18.44, 27.25, 30.38 mg/L (M2, M3 and M1), respectively. COD at locations M1 be was a low value due, M1 location, there was some refineries, but also was no waste that can cause high COD value, besides the water flow rate of M1 has compared the M4, so COD location M1 is not as high as precipitation M4. This is different from the causes of the high value of Ammonical nitrogen in Figure 4.7 because the rubbish contained on the site can decompose due to microbial activity in the soil or water.

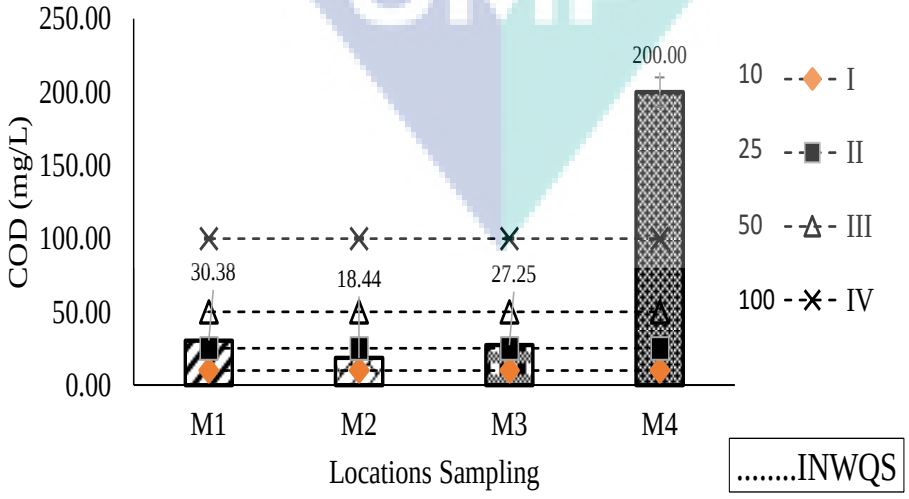


Figure 4.6: The Average Chemical Oxygen Demand (COD) values for 4 sampling point along of the Galing River water in Water quality standard

Locations M1, M2 and M3 in the industry Semambu area had also been a water flow rate, so that the waste does not undergo precipitation, while at the location m4 as the downstream area get piles of waste from upstream rivers where conditions are relatively clean even though located in the industrial area. Therefore, referring to the classification, Galing River is still proper and safe for domestic water supply and irrigation only if using extra water treatment.

4.2.6 Ammonical Nitrogen (NH_4-N)

In nature, Ammonia in the environment originates from metabolic, agricultural and industrial processes and from disinfection with chlorine (WHO, 2004). The concentration of Ammoniac Nitrogen (NH_4-N) data from four sampling points in Galing River was presented in Figure 4.7 which shows that the high Semambu-N concentration accounts for 3.42 semambu from M1 sampling point, M1 location semambu industrial area along the riverbanks many people are gardening and farming, from field observations of farmers also use pesticides to treat the plants, rainwater runoff that carries remnants of pesticides into water bodies Galing rivers. It is the result m1 location was the highest NH_4-N than other locations. While the lowest concentration of NH_4-N is 0.22 mg/L at M2 sampling point. The sample obtained from sampling point of the M3 and M4, the concentration is nearly the same level, which is at 2.16 and 2.57 mg/L respectively. This is causing M1 contradiction higher COD values in Figure 4.6, because the rubbish contained on the site can decompose due to microbial activity in the soil or water. Organic nitrogen early decomposed into ammonia, then oxidized to nitrite and nitrate. In the underground water and water contained in the surface, nitrate into compounds most frequently found. Because this occurs can be easily oxidized nitrite into nitrate. Contamination by nitrogen fertilizers, including anhydrous ammonia as well as organic waste, animal or human, can increase the levels of nitrates in the water. Compounds that contain nitrates in the soil are usually soluble and can easily migrate to ground water. It is also in accordance with the opinion (Sandrin and Maier, 2003 and Kuang, 2002).

Based on the standard of INWQS and DOE water quality index for Malaysia, M1 station sampling point is classified into class V, whereas sampling points of M3

and M4 fall into class IV. The other sampling point, M2 is categorized into class II. The highest concentration of NH_4-N is which is found at the M1 station in this study in agreement with the findings of (Mvungi et al., 2003) for a contaminated urban river in Zimbabwe.

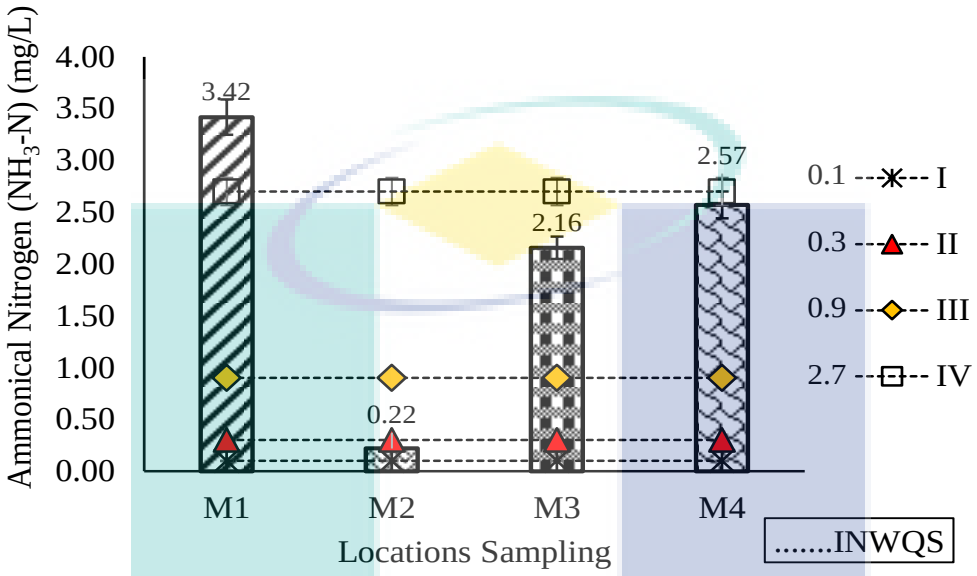


Figure 4.7: The average Ammonical nitrogen (NH_3-N) (mg/L) values of Four Sampling Point along Galing River Water for Water Quality Standard

4.2.7 Total Suspended Solid (TSS)

Total suspended solid for four sampling point stations is presented in Figure 4.8, It shows that the higher value of TSS is that for M1 (30 mg/L) sampling point station while TSS of the other sampling point stations, i.e. a value M3 and M4 is below 25 mg/L which has value of 12.9 and 4.5 mg/L, respectively. Based on the standard of INWQS and DOE water quality standard in Malaysia, M1 is classified into class II, whereas M2, M3 and M4 are ordered on class I. The high value of total solid in the water indicates to have high organic and inorganic pollutants absorbed in soils, so that the solids have high concentrations of the pollutant. These absorbed pollutants (and solids as carriers) can move elsewhere in water bodies, such as rivers and lake systems, causing organisms to be was exposed to pollutants far away from the point of source (Begum et al., 2009).

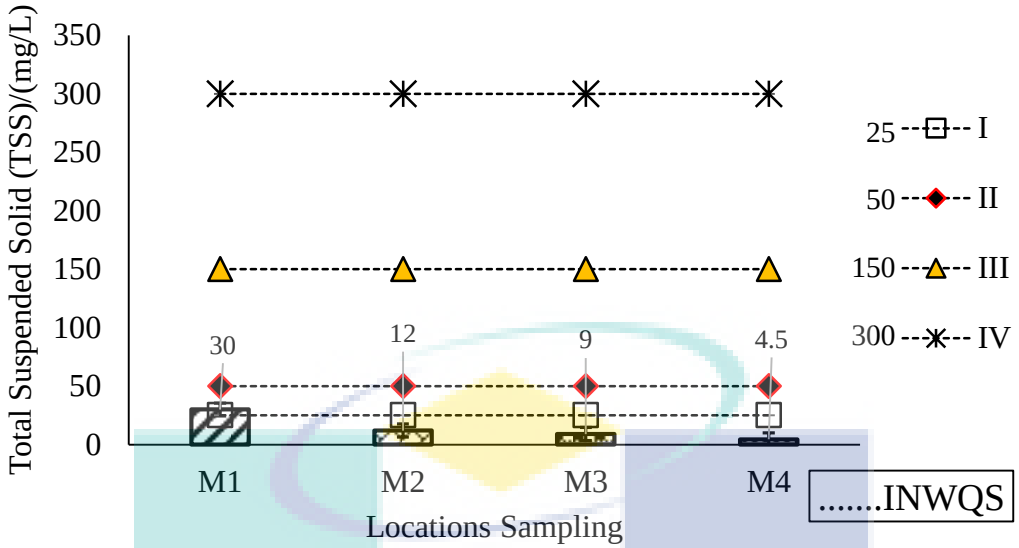


Figure 4.8: The average Total suspended solid (TSS) values for 4 sampling point along the Galing River water in Water quality standard

Analysis of total suspended solids in the water of the Galing River at four different sampling locations. All locations are included in class I except M1 sampling point which has category of class II which indicates that the river surrounding area has been cleared for building and premises. In addition, the riverside is not well maintained thus this condition causes soil erosion. Statement of research in a study (Buccolieri et al., 2006), that said condition caused high TSS value of various environmental factors, this is in accordance with the occur of environmental conditions.

4.2.8 Iron (Fe)

As presented in Figure 4.9, the highest concentration of iron is found to be 1.75 mg/L at M2 sampling point, followed by the figure of 1.52 mg/L at M3 and then 0.98 mg/L at M1 sampling point. The lowest concentration is found to be 0.65 mg/L at M4 sampling point. Based on The INWQS of Malaysia, M2 and M3 Sampling points are classified as class IV, while M1 point falls into class III, and the M4 is classified as class II. The highest concentration of Iron at M2 sampling point is due to industrial waste released from some existing industries surrounding the area, such as workshops, ice plant, and housing area. The level of iron concentration in all sampling points in Galing River is lower than that of Surma river which accounts for 3.16 mg/L as

reported by (Alam et al. 2007), while higher iron concentration as much as 0.04 ppb (part per billion) is in the Kernofully River (Majid and Sharma, 1999). Iron at location M2 was the highest value, we can refer to the location of the sample as in Figure 4.9, where this location form a basin that holds the waste water from various sources around the area M2. The sampling site conditions, the possibility of concentration of iron undergone various situations, both triggered by the environment or waste and microorganisms contained in the water.

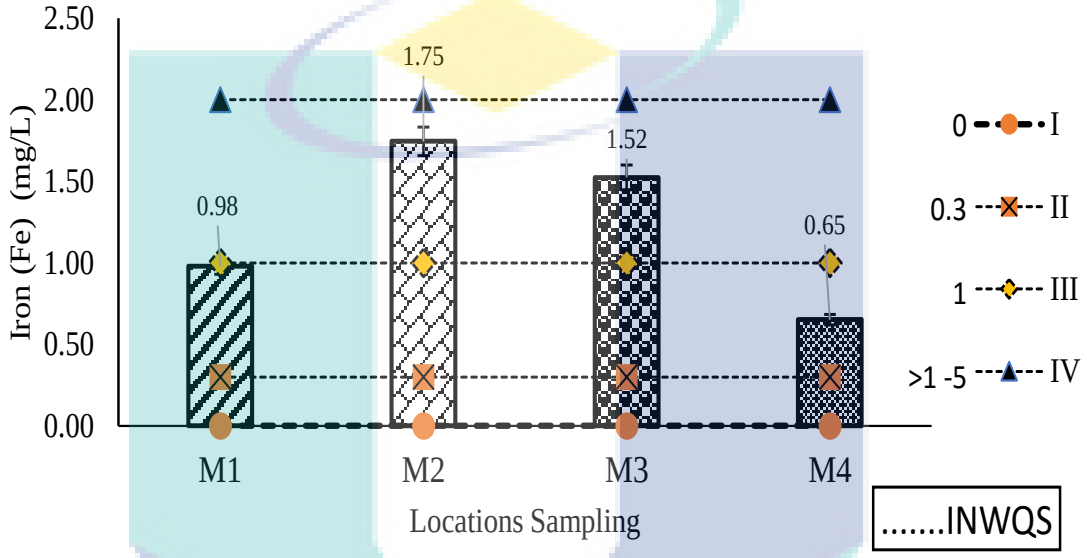


Figure 4.9: The average Iron (Fe) values for 4 sampling point along of the Galing River water in Water quality standard

4.2.9 *E. coli*

The total population of bacterial *E. coli* presented in Figure 4.10 shows, that maximum figure is 2,311.28 most probable number (MPN)/ml at the M2 sampling point. The figure of total *E. coli* population is almost similar to that at the M3 and M4 sampling points which account for 2202.95 and 2311.28 MPN/ml, respectively. On the other hand, the minimum bacterial *E. coli* population is as low as 1,483.43 MPN/ml at the M1 sampling point. Based on INWQS for Malaysia, all sampling points are classified into Class II. The temperature and effluent discharge into the river were effected to values of total *E. coli* in the water.

The moderate current of the Galing River stream also affects the population number of *E. coli*, in wet and dry periods. During drought periods, the high concentration results from the environment, providing little water discharge and solid waste. On the contrary, during rainy season and normal temperature condition, relatively large amount of water is abundant, in which, waste concentration is diluted. This result confirms with the finding reported by (Sabae et al., 2008).

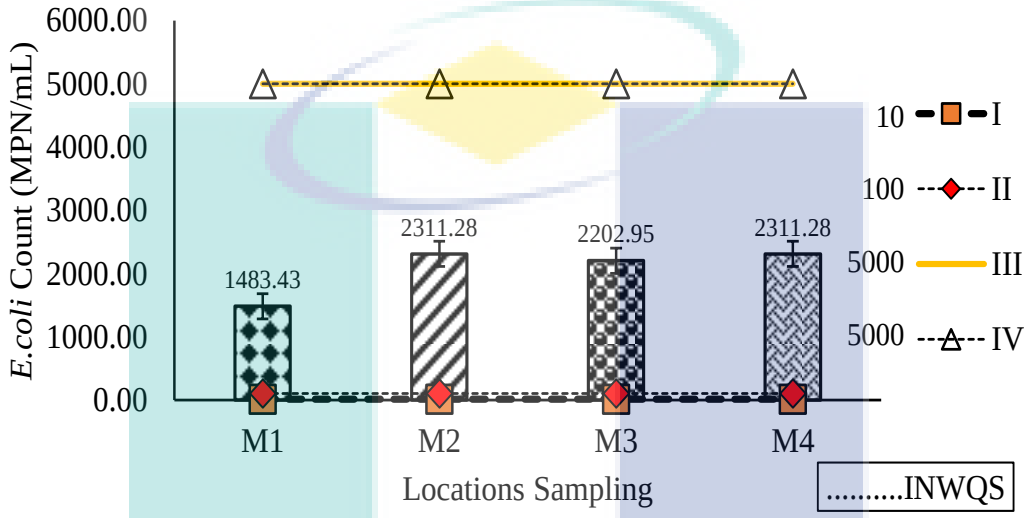


Figure 4.10: The average *E. coli* count for 4 sampling points along with the Galing River water in Water quality standard

4.2.10 Oil and Grease

Oil and grease is one of the important parameters that are used in water quality measurement. The data of oil and grease in four locations (M1-M4) are presented in Table 4.1 as can be seen the oil and grease content has increased from M1 to M4. The amount of oil and grease can be high or low depending on the water sample and the nature of the water. According to the calculation, using the (USEPA 10056) method of calculation, oil and grease final weight of the oil and grease content in the Galing river water was of 2-14 mg/L. The oil and grease content in M1 was low, since the major pollutant from M1 industrial are slaughtering of chicken. The river is not exposed to any factors that contribute to the content of oil and grease content in the water.

The sources of oil and grease in water can be caused by some factors such as petroleum refining, used oil refining and crude oil production. Furthermore, the oil

and grease content has increased in M2 and M3 due to the source of pollutant from both housing and workshop areas. In contrast the M1, M4 has higher oil and grease content, due to the pollutant in M4 which is from many sources (waste water from hotel, mall, workshop and housing), these pollutant has a lot of influence on the amount of oil and grease in the river water. In addition, this study waste cooking oil was chosen as representative oil and grease in the water. Based on the standard of oil and grease in river water, if the value of oil and grease is above 10 mg/L, it was classified as class IV. Station M2 and M3 of Galing River is classified as Class III, which is relatively clean. Meanwhile M4 is classified as Class IV which is slightly polluted.

Table 4.1: Oil and grease data from four sampling locations (M1-M4)

Sample	Oil and Grease (mg/L)
M1	2
M2	4
M3	7
M4	14

Based on the data in Table 4.1 the sampling location M4 shows it is of a high pollutant. From the initial observation of this location these is a need for treatments and bacteria isolation. Isolation of bacteria obtained from the sampling location, then grows and identified, further testing of bacteria was done to ascertain its ability to degrade WCO, which represents the number of waste contained in the Galing river water environment.

4.3 WATER QUALITY INDEX STANDAR CLASS OF GALING RIVER WATER

Water Quality Index (WQI) measures and evaluates river water quality. Initially, a water sample is tested in respect to the six chemical parameters and then referring to the National Water Quality Standards for Malaysia (NWQS), it is compared to classify their category.

Water Quality Calculation takes into account of the six parameters and then the result values are add into an established formula to obtain the WQI score:

WQI = 0.22 x SI DO + 0.19 x SI BOD + 0.16 x SI COD + 0.15 x SI AN + 0.16 x SI SS + 0.12 x SI pH

The value of water quality index (WQI) is determined by firstly obtaining the sub index value for each parameter. The WQI is a good indicator of any deterioration or improvement of a water body. Based on Figure 4.11 and Table 4.2, it shows that the sub index of WQI in sampling stations at M1, M2, M3 and M4 Galing River are 42.74, 55.16, 46.16 and 32.61, respectively. Among these four stations, sampling station of M2 indicates the highest value of WQI of all. This is mainly due to its surrounding where the fewest industrial activity exists, while the remaining stations are located for neighbouring with residential, industrial areas, and other land use. Based on the value derived from the sub index, station M2 of Galing River is classified as Class III, which is relatively clean and considered the least polluted. This is because surrounding of the river systems has a lot of trees that can help to reduce river pollution. Meanwhile, referring to the value derived from the sub index, location M1, M3 and M4 are classified as Class IV which is slightly polluted (Najah and Elshafie, 2009; and Mohamed, 2008).

Table 4.2: Status of River Pollution on Water Quality index (WQI) in Galing River

Station	si pH	si DO	si BOD	si COD	si SS	si AN	WQI	Status
M1	97.48	0.0	33.59	62.60	82.87	9.22	42.74	IV
M2	98.71	0.0	23.61	76.31	90.56	80.83	55.16	III
M3	98.54	0.0	14.94	67.59	92.21	39.53	46.16	IV
M4	99.29	0.0	56.42	52.42	94.81	21.28	32.61	IV

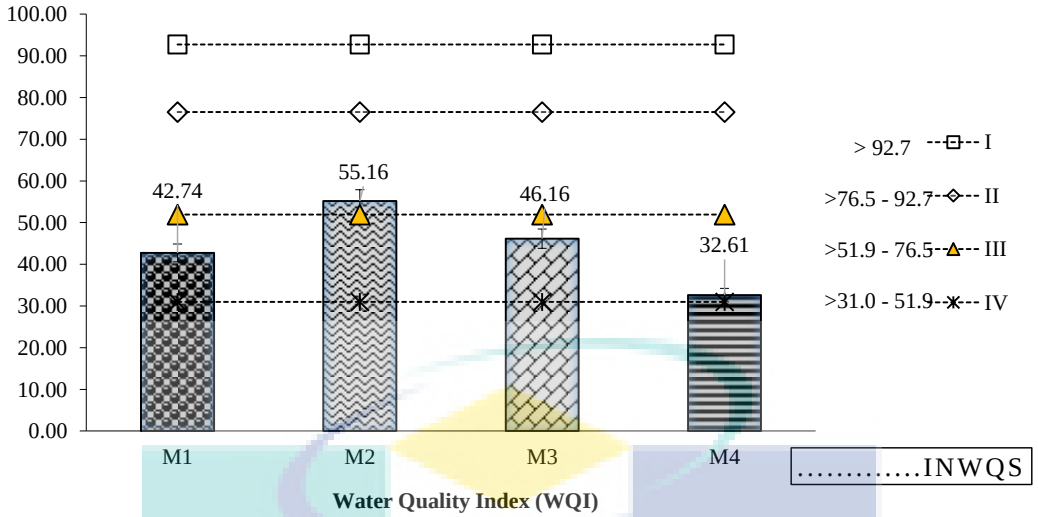


Figure 4.11: Water Quality Index of Sampling site of Galing River Performance of Different location area (M1) Street industrial semambu 4, (M2) Drain lorong setali 60, and (M3) Drain Street Galing 33, and (M4) downstream Vistana Hotel

From the above readings base on Figure 4.11 and Table 4.2, of the Galing River, the classification of WQI, assessment on Galing River by different sampling locations is classified into the Class III and IV. The sampling location of the M2 is grouped in the third class, while M1, M3 and M4 are categorized into the fourth grade. With respect to the grading of the river water (Munusamy, 2010), Galing river should only be used to support the organism of activities and the water should be used for livestock, drinking water and irrigation. In addition, the water is harmful for human needs and consumption, such as for drinking water, bathing, and washing.

The water quality index has multiple classes and terms, by which water is allowed to be utilized for specific purposes. From the results of the study, Galing River water can be assessed according to the standard as follows: Class I indicates that water body has an excellent quality. These standards meet the criteria for natural environmental conservation in its undisturbed state. Such water bodies are commonly found in areas of national park areas, fountain heads, undisturbed, and highland area. Within these undisturbed areas, any type or any kind of discharge is strictly prohibited. In addition, the quality of water bodies within this category complies with the strictest requirements for human health and protection of aquatic life (WHO, 2004).

Class IIA/IIB refers to water bodies which indicate good quality. Most sources of existing raw water supply are categorized into this class. In practice, to prevent water bodies from human pathogens that may occur, no activities involving body contact is allowed within in this water, Furthermore, it is required to establish another category of water bodies, to which is referred as Class IIB. The latter class of water bodies has the same quality yet it is not for water supply purpose. The standard determination for Class IIB is intended for the purpose of recreational function and aquatic protection for sensitive species.

Class III refers to water bodies to which, the aim is primarily to protect common aquatic species which are moderately tolerant and have economic value. In condition with extensive or advance treatment, water under this category is possible to be used for water supply. Water which belong to this class is also suitable for the needs of livestock and drinking water.

Class IV indicates that water quality can primarily meet the requirement for irrigation purpose of major agricultural activities, but does not cover minor applications for sensitive crops. Meanwhile, Class V refers to other waters which does not satisfy with any of the above criteria above (DOE 1974; EPA 1990; USEPA 1998)

4.4 ISOLATION OF BACTERIA FROM GALING RIVER WATER

The isolation of bacteria from Galing river water in a focus the location (M4) Downstream Vistana hotel, is based on the water quality index (WQI) This is location for pollutants, the results are shown in Table 4.2 and Figure 4.11, and Galing river water is classified into the class IV the range of 31.0 - 51.9 WQI. Based on the data, the location of the treatment is necessary to improve water quality of class IV into class II by research purposes. The degradation, resource that is used is difficult to disentangle the waste water in the waste cooking oil and oil & grease as shown in Table 4.1 the growing organisms is aimed at to get organism these to have a maximum organism's capacity in an effort to break down the waste to contaminated. This is why the place M4 location was chosen to isolate bacteria from the other three locations, namely (M1)

Street industrial Semambu 4, (M2) Drain Lorong Setali 60, and (M3) Drain Street Galing 33.

The isolation of bacteria from the Galing river water at the location M4 can be seen in Figure 4.12, the samples were first cultured on non-selective media Reviews such as nutrient agar (NA) as shown in Figure 4.12 (b) and nutrient broth method bacteria was used to determine the total bacterial population. Review of these bacteria were then selected in the media and was sub-cultured to obtain purely cultured bacteria as seen in Figure 4.12 an enumeration figure shows that the media with an enumeration of the total identified bacteria. After then isolation of bacteria from Galing river water samples by dilution 10^{-1} - 10^{-9} on location M4 with media growth nutrient agar (NA) was obtained. Then by the number the colonies of bacteria were counted (CFU/ml) using the plate count as shown in Table 4.3.

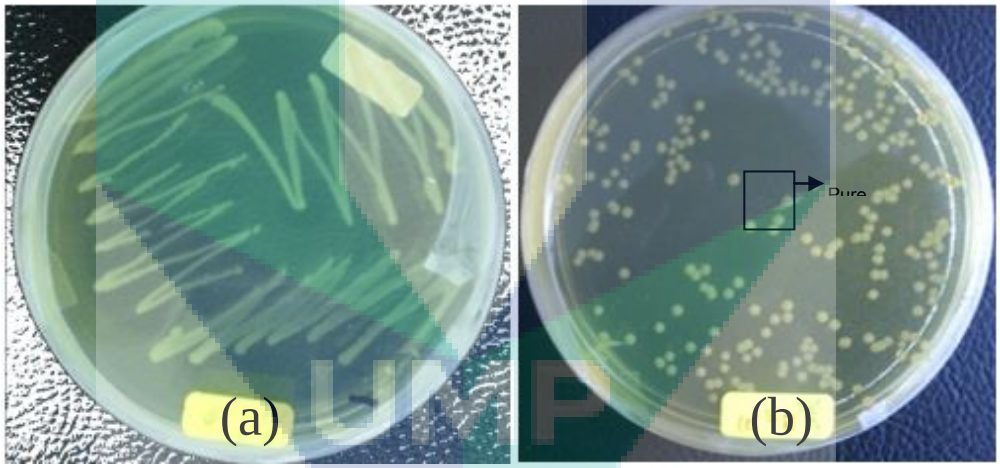


Figure 4.12: Isolation of bacteria from Galing river water on (a) pure culture bacteria and (b) culture bacteria

2.4.1 Determination of Bacteria by Quantifying Population Growth Colony Forming Unit (CFU/mL) and Optical Density (OD600 nm) in River Water Sample

The quantification of bacteria from the Galing River water has been carried out using plate counting method. The serial dilutions were plated out in order to achieve the desired range of colonies per plate (30 – 300). The total population of bacteria (CFU/ml river water) in site (M4) from downstream Vistana Hotel fluctuated from 1.7

x 10^{1} to 17 x 10^{7}. The total number of colonies is different from the dilution factor. The location M4 of the sampling area indicates that the content of bacteria in each sampling area is influenced by pollutants from the surrounding area (Reynolds et al., 2005). From the Table 4.3, it is shown that the CFU value of M4 (downstream Vistana Hotel) after dilution is at 10^{-1}, 10^{-3} and 10^{-8}. This is because the location of sampling points has a higher number of bacteria and was highly water polluted condition compared to M1, M2, M3 and M4 sampling points, according to the data of water quality index in Figure 4.11 and Table 4.2. The water conditions of contaminated the areas have enables bacteria types to live in the source of waste pollution. Population growth of colony forming units (CFU / ml) and the optical density had obtained 18 strains as shown in Table 4.4

Table 4.3: Bacterial Population with Dilution Factor in Agar Plate Count at location Sampling (M4) downstream of Vistana Hotel

Dilution factor	CFU/ml
	M4
10^{-1}	1.7 x 10^{1}
10^{-3}	1 x 10^{4}
10^{-8}	17 x 10^{7}

It is well recognized that some types of bacteria give benefit to the environment. Certain bacteria are able to live and have a role to improve the condition of the Galing River. Previous studies conducted by (Hamza et al., 2012; and Mukred et al., 2008), found that condition of food sources for bacteria derives from waste that can affect the growth and diversity of bacteria. This finding supports the research that has been done, where the sampling locations of Galing River have different types of waste and disparate environmental condition which has previously been explained in the analysis the index of national water quality standard presented in Table 4.2 and Figure 4.11. The population growth rate of bacteria is indicated by an increase in the cellular quantity and it is greatly dependent upon the nutrient availability in the environment. Measurement of turbidity or optical densities of the suspension of cells corresponds to the population of cells. Figure 4.13 Shows the various optical densities which is measured at 600 nm. These results indicate that the optical density of samples is correlated with the number of colonies per unit (CFU). The various optical density

values (OD) from dilution factor sampling location is presented in Figure 4.13. It depicts that the value of optical density at 10^{-1} and 10^{-3} are higher than that at 10^{-8}. This study proves that variation sample optical density (OD) is correlated with the number of colonies per unit (CFU) of bacteria in Galing river. There are some types of bacteria which have outstanding adaptability of growing in different conditions, as in the nutrient broth. It is in line with previous study than was reported by (Domde et al., 2007; Singh et al., 2013; and Ai et al., 2009).

4.5 SCREENING THE POTENTIAL LIPOLYTIC BACTERIA IN OIL BIODEGRADATION USING PHENOL RED, RHODAMIN B AND MACCONKEY

For screening potential of lipolytic activities, the qualitative methods have been widely used in several studies. Study of lipolytic bacteria was aimed to classify bacteria which have the ability to grow from location of sampling points M4. Thus, it is easier to identify later in applying bacteria to degrade waste cooking oil (WCO) in batch production.

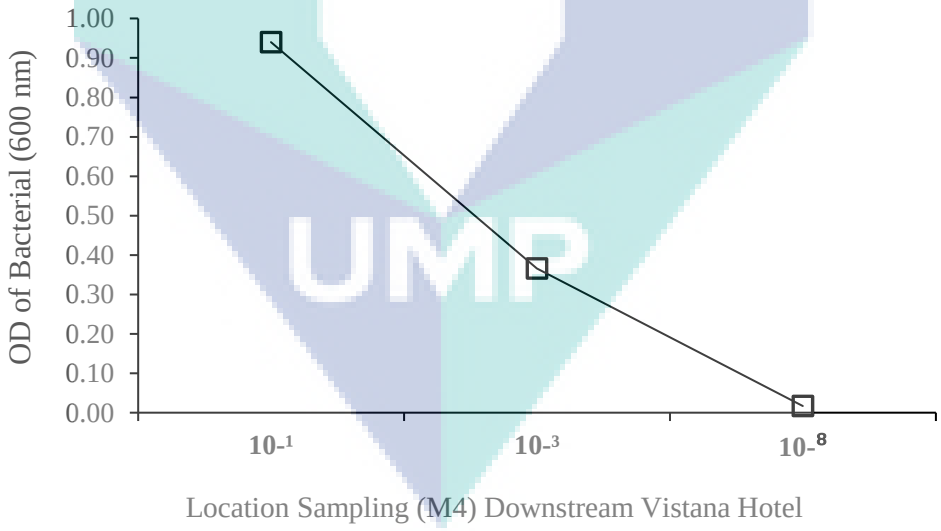


Figure 4.13: Optical density of bacteria at four site sampling (M4) downstream Vistana Hotel

In this study, only the bacteria population from M4 sampling point was used since it is based on the data on river water quality. The location M4 is highly polluted and it is predicted that bacteria obtained from these sampling locations are resistant, has

more survival and have a good ability in degrading waste material. The isolation of bacteria from the Galing river water are then tested using media selections as shown in Table 4.4. The bacterial growth singles colonies is derived from Figure 4.12 and the number of colonies forming unit (CFU) have been calculated.

The isolated samples of bacteria were screened using phenol red, Rhodamine B and MacConkey agar within three days of incubation, and then the microorganism has shown lipolytic activity. Phenol red has an end point at pH 7.3 - 7.4, which has red in color and a slight decrease in pH (7-7.1) when it turns to yellow. The yellow zone around culture indicates lipolytic activity. MacConkey agar is a specialized medium for bacterial growth This medium is selective for Gram-negative bacteria and it is able to differentiate those of gram bacteria which has the ability to ferment lactose (Kobayashi et al., 2012; Beauchamp et al., 2006; and Lee et al., 2010).

From the result Figure 4.13, it can be seen at 12 samples of isolated lipolytic from M4 totally exhibits the position samples of lipolytic using phenol red from total of 18 sample isolated bacteria. In the next step, the bacteria were tested using Rhodamine B and showed that only two of bacteria populations were identified to have positive lipolytic activity. These samples consist of bacteria N and Q. The microbial colonies with positive lipolytic bacteria indicate an orange fluorescence and continuing incubation time. Orange fluorescent were about formed around the colonies (Arku et al., 2012; and Lee et al., 2010). Meanwhile, by continuously testing using MacConkey Agar, only 5 samples of isolated bacteria were not identified to indicate lipolytic bacteria, i.e. sample bacteria of H, Q, 3, 4 and 8. Screening using MacConkey agar plates shows color change in the media which it turns into dark brown around the organism. The change of color in each test using phenol red, Rhodamine B and MacConkey is presented in Figure 4.14 and Table 4.4.

Table 4.4: Test lipolytic bacteria to degraded waste cooking oil with Phenol Red, Rhodamine B and MacConkey Agar

No	Name Sample	Phenol Red	Rhodamine B	MacConkey Agar	Location Sampling
					M4
1	G	-	-	-	√
2	H	++	-	+	√
3	J	++	-	-	√
4	K	-	-	-	√
5	M	-	-	-	√
6	N	++	++	-	√
7	O	-	-	-	√
8	P	++	-	-	√
9	Q	++	+++	+	√
10	S	++	-	-	√
11	T	++	-	-	√
12	U	++	-	-	√
13	1	++	-	-	√
14	2	++	-	-	√
15	3	++	-	+	√
16	4	-	-	+	√
17	6	++	-	-	√
18	8	-	-	+	√

*Note: (-) Not Detected, (+) Poor, (++) Moderate and (+++) High.

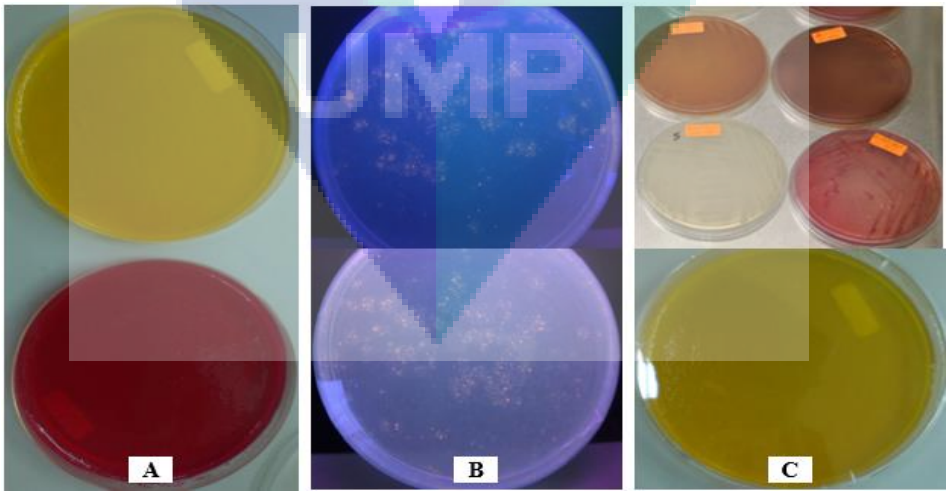


Figure 4.14: (A) Phenol Red, (B) Rhodamine B and (C) MacConkey

After the selection lipolytic bacteria in selective media (phenol red, Rhodamine B and MacConkey), the bacteria were identified using gram straining to recognize their shape and characteristic (+) or (-). Finally, the bacteria were then analyzed using Bergey's manual.

4.6 CULTIVATION AND MORPHOLOGYCAL CHARACTERIZATION OF BACTERIA ISOLATES

Isolation and identification of bacteria as shown in the Table 4.5 is to determine the morphology and characteristics of bacteria using Gram straining. Total bacteria were from 18 samples obtained from locations of sampling point M4. Several morphological forms of bacteria were detected, namely: *Streptococcus, Bacilli, Streptobacilli, Diplococcus Coccus, Cocobacilli* and *Staphylococcus*. A type of bacterium that is obtained during the isolation of river water from of different locations, have a tendency to form bacilli in term of bacterial morphology, while the remaining two forms different shapes, such as *Streptococcus, Streptobacilli* has two forms, and *Diplococcus* has two forms, each remaining coccus, *Coccobacilli* and *staphylococcus* has one form. The bacterium of type Bacilli was found to be the majority in Galing River water. These bacteria belong to bacteria, pathogens. A high population of Bacilli found in Galing river water. Indicates that the water is not safe for a consumption and cooking. The isolated bacteria of Bacilli from river water were reported in previous work conducted by Olayemi et al. (2008). The industrial waste contributes positively to bacteria growth in the river water.

The identified bacteria using Gram stain was found to be of 5 Gram (+) and 13 Gram (-) with a total of 8 bacteria isolated from M4 sampling point. The characteristic shape of bacteria is a variation of 7 convex, 1 irregular, spindle 2, circular 2 and 6 raised. We can observe that the irregular shape and convex has the same number of bacteria in the dominating characteristic, while the next value of the 6 raised, then 2 circular and 2 spindles. The margin of isolated bacteria was also investigated. It is found that the major margin bacteria isolated are lobate. From the Table 4.5, based on shape and characteristic Gram straining, the isolated bacteria are classified into eight groups with code grouping (A-H).

The Gram reaction screening to the isolated sample was carried out. The isolates were identified and grouped into eight types of bacteria, namely A, B, C, D, E, F, G, and H. (Table 4.5). Based on Table 4.6, the strain all group can be found in sampling point. The Screening isolates of Gram staining of stain group A-H is shown in the Figure 4.15. The morphology of bacteria differs from morphology and Gram stain. The bacteria could be identifed.

Table 4.5: Isolation of bacteria from M4 areas of Galing River water the Gram straining reaction and characteristic with the Gram staining reaction

No	Isolates Code	Isolates Colony		Gram-stain Reaction	Elevation	Margins	Sample Group
		Color	Shape/Form				
1	G	Pink	Bacilli	-	Spindle	Felamentous	(E)
2	H	Purple	Streptobacilli	+	Circular	Lobate	(F)
3	J	Pink	Bacilli	-	Raised	Felamentous	(G)
4	M	Pink	Bacilli	-	Raised	Undulate	(E)
5	N	Pink	Bacilli	-	convex	Lobate	(E)
6	O	Pink	Bacilli	-	Spindle	Felamentous	(E)
7	P	Pink	Streptococcus	-	convex	Lobate	(A)
8	Q	Purple	Coccus	+	convex	Lobate	(C)
9	R	Pink	Streptococcus	-	Irregular	Lobate	(B)
10	S	Pink	Bacilli	-	convex	Lobate	(A)
11	T	Pink	Bacilli	-	convex	Lobate	(A)
12	U	Pink	Steptobacilli	-	Raised	Felamentous	(G)
13	1	Pink	Bacilli	-	Raised	Felamentous	(G)
14	2	Pink	Bacilli	-	convex	Lobate	(A)
15	3	Purple	Cocobacilli	+	convex	Lobate	(D)
16	4	Purple	Bacilli	+	Raised	Undulate	(H)
17	6	Pink	Diplococcus	-	Circular	Lobate	(E)
18	8	Purple	Staphylococcus	+	Raised	Undulate	(H)

The strain name of isolated bacteria was identified using Bergey's manual. Based on the shape and characteristic of bacteria collected from M4 areas of sampling points at Galing river, is shown in the Table 4.6 and Table 4.7 and presented in Figure 4.15, eight types of the bacteria are identified, namely *Pseudomonas* sp, *Providencia* sp, *Shigella* sp and *Escherichia coli* which are classified as Gram negative. Meanwhile, *Salmonella* sp, *Staphylococcus* sp, *Bacillus* sp and *Clostridium* are classified as Gram positive. On the other hand, the M4 sampling points are identified to be a residence of different type of bacteria. This is probably due to condition of surrounding area nearby

sampling point which supports different type of bacteria to grow well (Rahman et al., 2002; Senthil et al., 2011; and Shokrollahzadeh et al., 2008).

Table 4.6: Identification of Lipolytic bacteria from Galing river water at (M4) downstream Vistana Hotel,

No.	Name Strain Group	Number Isolate	Characteristic	
			Gram-Reaction	Shape/Form
1	*A*	P,S, T,2	Negative	Rod
2	*B*	R	Negative	Cocci
3	*C*	Q	Positive	Rod
4	*D*	3	Positive	Cocci
5	*E*	G, M, N, O, 6	Negative	Rod
6	*F*	H	Positive	Rod with endospores
7	*G*	J, U, 1	Negative	Shoat rods
8	*H*	4 and 8	Positive	Rod with endospores

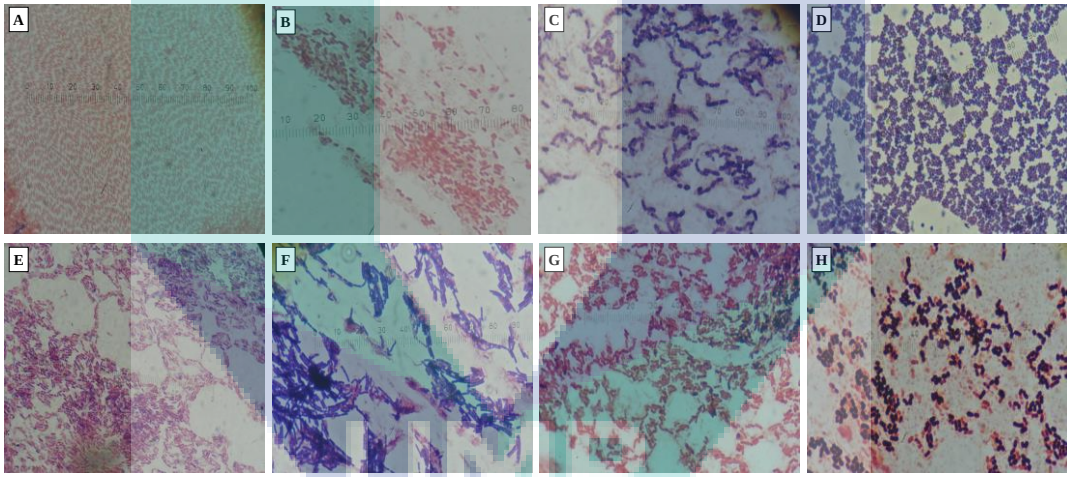


Figure 4.15: Identification stain from Galing river water locations sampling (M4) downstream Vistana Hotel

Table 4.7: Identification of bacterial strains in (M4) Downstream Vistana Hotel location sampling Galing River Water

No	Name Strain Group	Number Isolate	Strain	Gram	Shape/Morphology
1	*A*	P,S, T,2	*Pseudomonas sp*	Negative	Rod
2	*B*	R	*Providencia sp*	Negative	Cocci
3	*C*	Q	*Salmonella sp*	Positive	Rod
4	*D*	3	*Staphylococcus sp*	Positive	Cocci
5	*E*	G, M, N, O, 6	*Shigella sp*	Negative	Rod
6	*F*	H	*Bacillus sp*	Positive	Rod with endospores
7	*G*	J, U, 1	*Escherichia coli*	Negative	Shot rods
8	*H*	4 and 8	*Clostridium*	Positive	Rod with endospores

4.7 ESTIMATION OF BACTERIA GROWTH POTENTIAL TO WASTE COOKING OIL (WCO) DEGRADATION

To determine the growth of potential bacteria isolated to degrade waste cooking oil (WCO), and measured by OD analysis the bacteria isolated from an M4 total of 18 isolates and are classified into eight groups shown in Table 4.5 and Table 4.6. For 4 days the bacteria growth was isolated in enrichment solution, and then measured by UV spectrophotometer to observe OD each day. Figure 4.16 shows that the growing the first day isolated from M4 had increased from first day to the second day, while with additional days of growing the OD value had decreased. The higher OD value indicates higher rates of bacteria growth in the media. It reveals that the bacteria isolated from M4 appears to increase significantly. This result is in agreement with the previous study reported by (Oboh. et al. 2006), where the OD value decreased by increasing the time of incubation due to exponential growth of bacteria in the media.

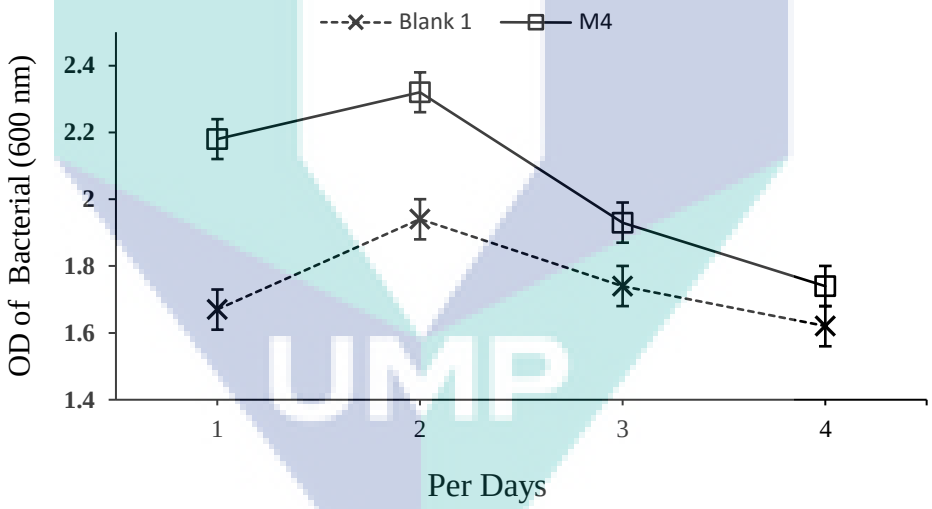


Figure 4.16: Measuring optical density per days with locations sampling M4 to estimation of bacterial growth potential to degrade WCO

After isolating bacterial its measured Optical density, then isolates from Galing river water for their ability tested to degrade waste cooking oil by means of degrading a carbon source on filter paper in a petri dish. As in the Figure 4.17.

4.8 TESTING THE ABILITY OF BACTERIA TO DEGRADE WCO AS CARBON SOURCE

The ability of isolated bacteria to degrade WCO as a carbon source in filter paper was investigated in this study. The bacteria isolated was spread evenly on a petri dish, and then placed on filter paper containing WCO. As shown in Figure 4.17 the clear zone in filter paper was observed, which indicated that WCO was degraded by isolated bacteria after being incubated for three days.

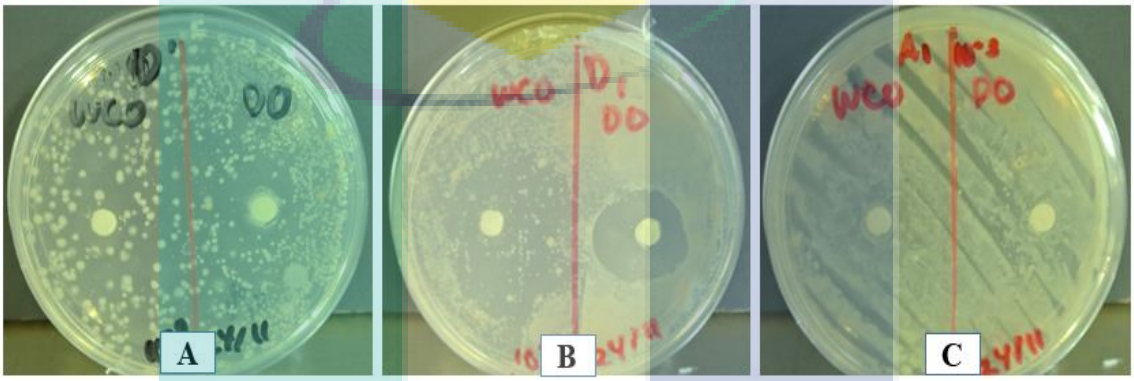


Figure 4.17: Isolate Bacteria in nutrient agar (NA) to see degrade WCO as carbon source with zone filter paper analysis (A) poor, (B) moderate and (C) height

The small clear zone of filter paper is an indicator that the isolated bacteria have the ability to degrade WCO. This result confirms with the study conducted by (Shalaby, 2011; Hamme et al., 2003; Abdel-Raouf et al., 2012; Oboh et al., 2006; and Wang et al., 2012). On the other hand, the wide clear zone of filter paper is an indication that isolated bacteria have lower ability to degrade petroleum as a source of carbon. Table 4.8 shows that all 18 isolated bacteria as the same from Table 4.4 and are able to degrade WCO as a carbon source. Meanwhile, each of the bacteria exhibit has different abilities to degrade WCO. The lowest ability to degrade of WCO is attributed to bacteria in the sample Q, 3, 4, and 8.

Table 4.8: Test the ability of lipolytic bacteria to degraded waste cooking oil as carbon source with zone filter paper analysis

No	Name Sample	Zone Clear Petri Dish	Sample Group	Location Sampling
				M4
1	G	+++	(E)	√
2	H	+++	(F)	√
3	J	++	(G)	√
4	M	+++	(E)	√
5	N	++	(E)	√
6	O	+++	(E)	√
7	P	+++	(A)	√
8	Q	+	(C)	√
9	R	+++	(H)	√
10	S	+++	(A)	√
11	T	+++	(A)	√
12	U	+++	(G)	√
13	1	++	(G)	√
14	2	++	(A)	√
15	3	+	(H)	√
16	4	+	(H)	√
17	6	+++	(E)	√
18	8	+	(H)	√

*Note: (-) not detected, (+) poor, (++) moderate and (+++) high.

4.9 IDENTIFICATION OF BACTERIA WITH RAPID ONE TEST

The recent invention pertaining to culture media, which is useful for RapID screening (shown in Figure 4.18) in the field of clinical and environmental cultures in order to detect some of the most common pathogenic bacteria. The observation of reactions to these media is meaningful for rapid and economical presumptive diagnoses of infection that caused by various bacteria. This method is also useful for screening food and water samples for infection prevention. Even though various additional applications are being assessed, these useful media are particularly being used for testing samples which probably contains enteric organisms such as *E. coli* and *Salmonella* species (Widjojoatmodjo et al., 1994).

Testing of samples, bacteria using RapID one test were from isolates derived and then tested using test the ability of bacteria to degrade the waste cooking oil using a carbon source in the filter paper as in Figure 4.17 and Table 4.8. The isolates has a total 14 from 18 isolate, because of it is rapid, only one test has 14 field trials, thus only 14 a

selected isolates were tested as in Figure 4.18 and table 4.8. The isolates were 4 while non-selected H, Q, R, and 3, then going into tests using DNA. This is because in addition to the limited test kits, chemical test is also very sensitive and isolates these bacteria have a different character to the others, as the composition is more dense and dry so it is difficult to react. The accuracy of rapid one test on several samples that have been tested not using rapid one test will be tested using DNA extraction.

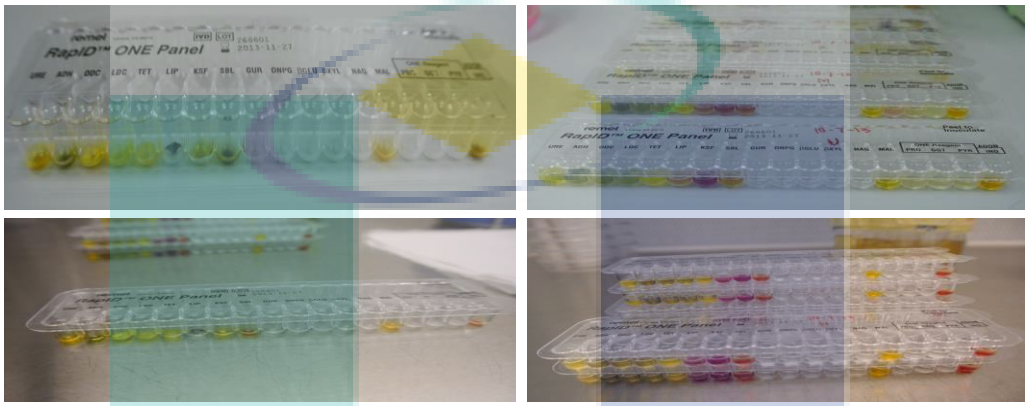


Figure 4.18: RapID One Panel testing bacteria

The identification of bacteria using RapID one, were investigated and tabulated in Table 4.8. From the Table, it shows that the major bacteria identified is *Shigella sp,* which is found in 9 samples stain from Galing River. Bacteria *Shigella sp.* belongs to pathogenic types which can cause diseases for human. The colonization of the gastrointestinal tract by these pathogens may lead to diseases, for instance bacillary dysentery caused by *Shigella*, diarrhoea, which causes *Salmonella*, typhoid, and so on. Many of the bacterial-related diseases are associated with the gastrointestinal tract which are caused by ingestion of pre-formed toxins which are from contaminated foods, such as toxins produced by *Staphylococcus aurous, Bacillus cereus, Clostridium botulinum*, etc. Furthermore, numerous organisms which have infected the gastrointestinal tract will release toxins causing the signs and symptoms of disease, such as cholera, *pseudomembranous colitis* associated antimicrobial due to *Clostridium*, etc. A recent study found that some stereotypes of *E. coli*, which are commonly harmless commensal organism residing in the mammals' intestinal tract have been acknowledged as important pathogens (Stabili and Cavallo, 2011).

Testing of bacterial isolates from Galing river was water done using one rapid test Table 4.8 shows the number of pathogenic bacteria of the type of *Shigella* sp. This is in accordance with the water quality index that had been of the previously analyzed in the river as shown in Figure 4.11. The Galing water quality is at the fourth grade, while the types of bacteria tests using a rapid one test had detected that *Moellerella wisconsensis*, *Leminorella grimontii* (EG 57), *Providencia stuartii*, and *Acinetobacter calcoaceticus*, isolates obtained 4 types from 18 samples. There are four isolates that were not detected, namely H, Q, R and 3. To confirm the accuracy RapID ONE test several sample that have been tested not using RapID ONE test, were tested using DNA extraction. While six samples were selected from the results of the RapID One test, that was samples 4, 6 and O is a sample that was more probability of 99.9%, while the three others, namely J, M and N is a probability sample of its 62.18%, this RapID ONE test was to confirm the test in identifying further using DNA extraction including 4 samples that were not detected by the test.

Table 4.8: Identifications of Bacteria with RapID One specialization detection for Gram (-) bacteria from Sample Galing River water

No	Name Sample	Name Stain	Microcode	Probability
1	G	*Shigella sp*	0200400	> 99.9%
2	J	*Shigella sp*	0200000	62.81%
3	M	*Shigella sp*	0200000	62.81%
4	N	*Shigella sp*	0200000	62.81%
5	O	*Shigella sp*	0200400	> 99.9%
6	P	*Moellerella wisconsensis*	0000040	> 99.9%
7	S	*Shigella sp*	0000000	95.39%
8	T	*Shigella sp*	0200000	62.81%
9	U	*Shigella sp*	0200000	62.81%
10	1	*Shigella sp*	0220000	99.43%
11	2	*Moellerella wisconsensis*	0000040	> 99.9%
12	4	*Leminorella grimontii* (EG 57)	0200110	99.77%
13	6	*Providencia stuartii*	2200110	> 99.9%
14	8	*Acinetobacter calcoaceticus*	2200000	94.65%
15	H	*Not detection*	-	-
16	Q	*Not detection*	-	-
17	R	*Not detection*	-	-
18	3	*Not detection*	-	-

4.10 PCR AND DNA (ANALYSIS OF 16S rDNA GENE SEQUENCES AND PHYLOGENETIC ANALYSIS)

4.10.1 Genomic DNA Extraction

DNA sequencing consists of a number of methods and technologies which were used to determine the nucleotide base-adenine, guanine, cytosine and thymine in a DNA molecule. In spite of a time consuming method, DNA sequencing is powerful tool since it allows scientists to read the nucleic making up of individual genes. Sequencing information is essential for gene cloning and genetic manipulation (Aghapour et al., 2013).

The identification of isolated bacteria using DNA extraction method was presented in Figure 4.19. From the figure, it is shown that DNA was initially extracted from all samples counting totally for twelve isolated bacteria. These results are supported by the gel electrophoresis result. The figure of gel electrophoresis indicates the representative sample (N, 6, Q, H, J, M, O, 4, 3 and R) which were previously isolated from Galing river water.

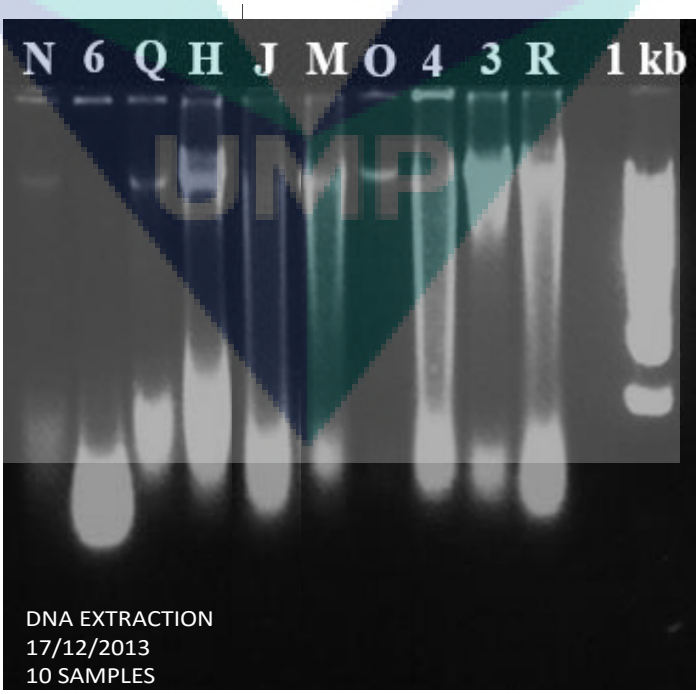


Figure 4.19: Aga-rose gel electrophoresis of DNA extraction

Strain samples 10 of isolates were selected based on preliminary information that was obtained from rapid one test sample of N, 6, J, M, O and 4, as well as to confirm its accuracy. While the sample Q, H, 3 and R was derived from testing the ability of bacteria to degrade a carbon source such as in Table 4.8. Of all samples tested isolates using DNA Extraction is expected to represent of 8 types group of bacteria obtained from the isolation of bacteria from Galing river water as shown in Table 4.5.

4.10.2 PCR Amplification with Universal Primers and PCR Product Purification

Biophotometer reading on the concentration of bacteria DNA extraction is tabulated in Table 4.9. The spectra was previously measured using UV spectrophotometer upon the light absorbance in the wavelength region of 230 nm to 320 nm. A good quality DNA extraction sample could be identified from this Table. The purity of sample should exhibit a ratio of absorbance at 260 nm over 280 nm or A_{260}/A_{280} of 1.7 to 2.0 and the value of A_{260}/A_{230} ratio is above 1.5 (Rasko, 2004). From the Table 4.9, five of sample stains (consist of sample 6, Q, H, 3 and R) are categorized in these ranges. Then, the selected bacteria samples are sent for PCR sequencing analysis. It is based on analysis results of age-rose gel electrophoresis, which states that the sample 3, H, 6, Q and R are the best sample purity for approaching pure culture and is eligible to be tested using sequencing PCR analysis.

Table 4.9: Biophotometer reading the concentration of bacterial DNA extraction

No	Sample Name	Yield (µg/ml)	Purity (Average Yield)	
			A260/A280	A260/A230
1	H	45.4	2.07	1.48
2	J	26.0	2.05	1.44
3	M	14.3	1.97	0.64
4	N	6.5	2.11	0.25
5	Q	12.3	2.03	0.81
6	R	35.6	2.03	0.54
7	O	2.6	1.94	0.17
8	3	15.5	2.01	0.64
9	4	43.7	1.97	0.51
10	6	39.1	2.01	1.54

4.10.3 Electrophoresis Visualisation of Extracted DNA and PCR Products

Figure 4.20 illustrates the amplified PCR products sequencing extraction 16S rRNA, resembling the approximate size of 600 bp. The gel electrophoresis pictures indicate the representative five samples (3, H, 6, Q, and R) isolated from Galing river water. All of the representative samples were successfully amplified from bacteria DNA, which are found in the samples. Samples which were sent for the subsequent sequencing method were therefore selected based on successful amplification with either primer set. Out of ten DNA extraction samples, only five selected samples were sent for PCR analysis. The list of bacteria which were identified using PCR analysis are tabulated in Table 4.9. The underlying chosen five samples 3, H, Q, 6 and R, it is a good quality DNA extraction sample that could be identified from this Table 4.9 and Figure 4.19. And the purity of the sample should exhibit a ratio of absorbance at 260 nm over 280 nm or A_{260}/A_{280} of 1.7 to 2.0 and the value of A_{260}/A_{230} ratio is above 1.5. If the quality of DNA extraction is not good or less than the provisions, it will be difficult to do PCR analysis.

4.10.4 Sequence Analysis and Blast Analysis

The list of bacteria which have potential lipolytic degradation is identified using DNA sequencing and were tabulated in Table 4.10 the five potential bacteria were successfully identified, i.e. *Providencia stuartii*, *Bacillus pimilus, Exiquobacterium* sp. and *Bacillus antracis. Ames.* Out of the five bacteria isolated, only two types of bacteria which is potential (stain 3 and R), namely *Bacillus antracis. Ames*. The stain 6 was identified as a *Providencia stuartii*. This study result concurs with results using RapID One (presented in section 4.9).

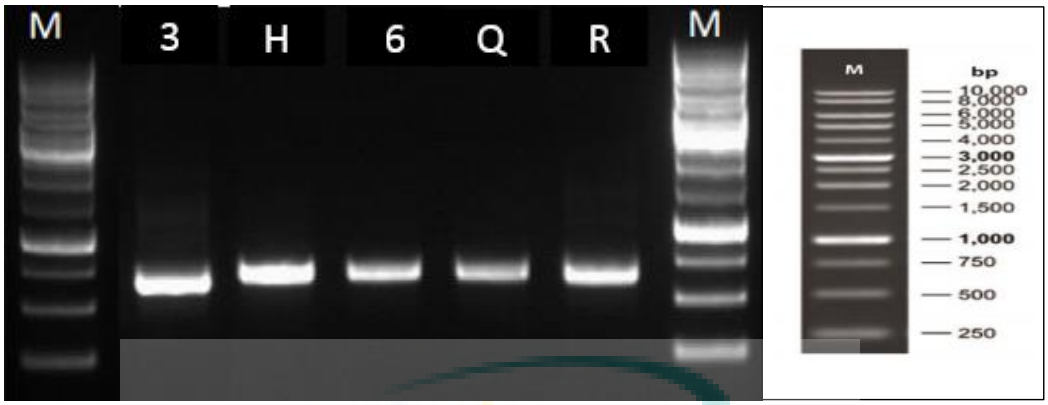


Figure 4.20: Gel photo PCR sequencing extraction 16S rRNA, ~ 600 bp partial

The phylogenetic trees of sample of bacteria code (6) is depicted in Figure 4.19. The bacteria identified from this sample, *Providencia stuartii* belongs to one of various species of Gram negative. According to Odeyemi et al. (2013), bacteria of Gram-negative are likely to have more resistance to various lipophilic as well as amphiphilic inhibitors compared to bacteria from Gram-positive.

After having the four bacteria identified using DNA sequencing is used of a laboratory scale to degrade the waste cooking oil, using the formula gravimetric and FTIR analysis. All bacteria were tested in variations of 1-4% waste cooking oil with the addition of bacterial isolates variation of 50. 75 and 100 µl, for four days at a temperature of 30 ^{0}C in incubator shaker. This can be seen in Figure 4.26 and Figure 4.28 below.

Table 4.10: Identification bacterial potential degradation lipolytic using DNA sequencing from sample Galing river water

No	Name Sample	Results	Test Method
1	6	*Providencia stuartii*	Sequencing (out-source)
2	Q	*Bacillus pimilus*	Sequencing (out-source)
3	H	*Exiquobacterium sp.*	Sequencing (out-source)
4	3	*Bacillus antracis. Ames*	Sequencing (out-source)
5	R	*Bacillus antracis. Ames*	Sequencing (out-source)
6	N	Done until DNA Extraction only	
7	J	Done until DNA Extraction only	
8	M	Done until DNA Extraction only	
9	O	Done until DNA Extraction only	
10	4	Done until DNA Extraction only	

The genus *Providencia* includes urease, which are responsible for a wide range of infectious diseases to human. In spite of the fact that most infections are caused by *Providencia* which entail the urinary tract, these infections also relate to gastroenteritis and bacteremia. However, their presence in a water system could lead the major health concerns and may indicate the presence of potential disease-causing bacteria stains, i.e. pathogens (Paulse et al., 2012). Both of the species (*Providencia rettgeri* more occurring than *Providencia stuartii*) have recently been identified as etiologic agents in traveler's diarrhea, making travel history important in patients with acute diarrhea and urinary tract. A common symptom in gastrointestinal infection caused by *Providencia rettgeri* is vomiting. Very commonly, patients who have burn injuries are more prone to wound infection by *Providencia* (Ash et al. 1993; Huo et al. 2010; Kafilzadeh. et al. 2010). The nine species are currently in the genus *Providencia*, descending order of phylogenetic tree, including *Providencia stuartii*, *Providencia sneebia*, *Providencia vermicola*, *Providencia rettgeri*, *Providencia heimbachae*, *Providencia burhodogranariea*, *Moellerella wisconsensis*, *Providencia alcalifaciens* and *Providencia rustigianii*.

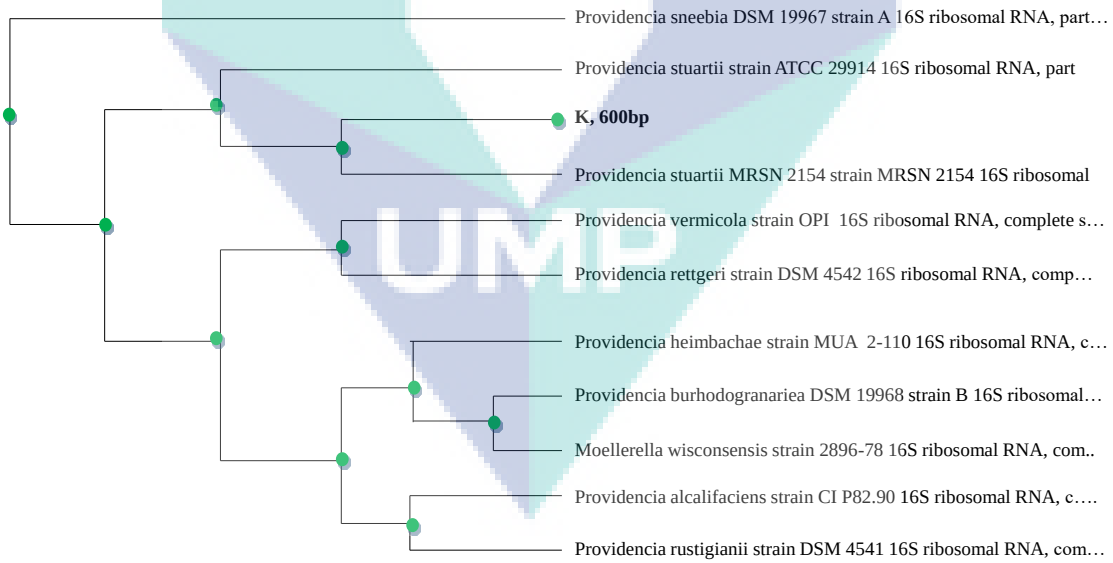


Figure 4.21: Phylogenetic Bacteria code (6) *Providencia stuartii* by NCBI Blast Tree Method

One of the isolated sample of bacterial (Q) species is identified as the organism, *Bacillus pumilus*, belonging to Gram positive. The phylogenetic trees of which is depicted in Figure 4.22. A number of biochemical assays which are found in the

analytical profile index have been used to determine its classification. Although *Bacillus pumilus* is amylase, lipase, and protease-positive, this bacterium has various mechanisms to reduce nitrate, produce gas from glucose, and produce acid from various sources of carbon, such as arabinose, xylose, glucose, mannitol, and lactose. Meanwhile, bacteria *B. pumilus* can remarkably manufacture acetylbutanediol from acetone, as indicated by a positive result in the Voges-Proskauer test (Xiao, 2009). Acetylbutanediol belongs to a class of diols which have a significant role in synthesizing numerous polymers by microbes and also have potential use for alternative fuel to substitute conventional fossil fuel feedstock. It confirms with previous study conducted by Paulse et al. (2012), which studied Berg river in South Africa. Some of Gram positive bacteria consist of various *Bacillus sp* including *Bacillus pumilus Staphylococcus sp*, and the *Firmicutes* bacterium. All these bacteria are identified to be present in this river.

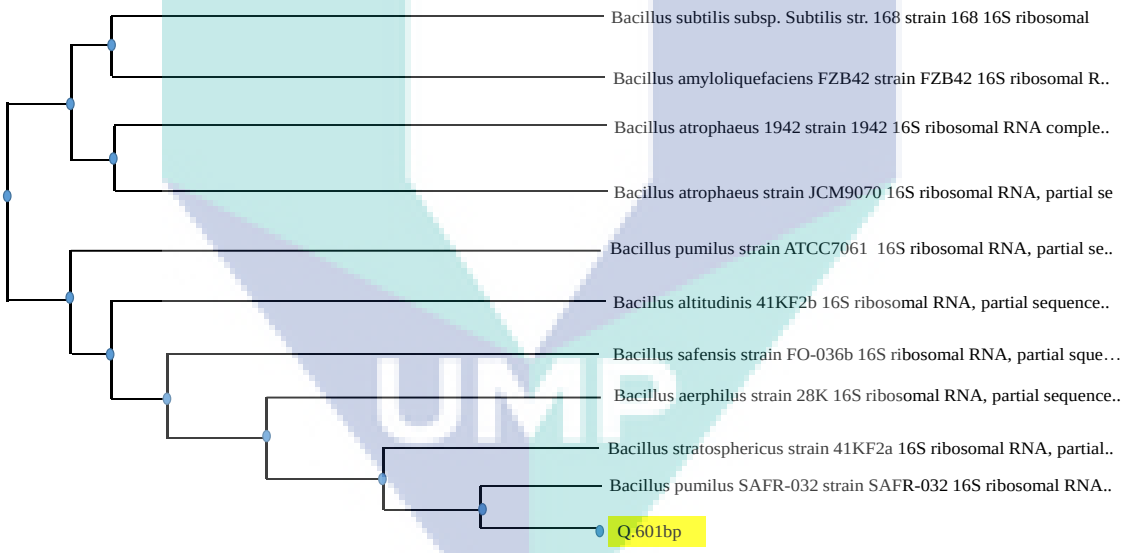


Figure 4.22: Phylogenetic of Bacterial isolate (Q) *Bacillus pimilus* by NCBI Blast Tree Method

The phylogenetic trees of bacteria of the genus *Exiguobacterium* sp. are presented in Figure 4.23 Their cells are rod-shaped and these bacteria belongs to Gram-positive facultative anaerobes that have been repeatedly isolated since very long time.. Some researchers reported that *Exiguobacterium sp* exhibits a great potential in wide range of industrial applications, such as for wastewater treatment, biotechnology, bioremediation agents, and agricultural applications. *Exiguobacterium* strain Z8 has an

ability to neutralize highly alkaline wastewater treatment discharged from textile industry (Kumar et al., 2006). In addition, Buthelezi (2008) also reported that bacterial Bioflocculants, such as *Pseudomonas plecoglossicida, Staphylococcus aureus, Pseudomonas pseudoalcaligenes, Klebsiella terrigena, Bacillus subtilis,* and also *Exiguobacterium acetylicum,* were investigated on their potential to remove dyes contained in textile wastewater as well as to reduce the microbial load in untreated river water. Bioflocculants are substance of extracellular polymers which are synthesized by organisms or living cells. Dhanve et al. (2009) reported in the study that isolated *Exiguobacterium sp* required as long as 48 hours for decolorizing sulfonated hike dye Navy blue HE2R. In the study, *Exiguobacterium sp*. MG2 was isolated from a river in Yunnan Province of China as one of the best to degrade malachite green (MG). One of the most commonly used dyes is in textile industry, N-methylated diaminotriphenylmethane has effectively been used for an antifungal agent. Due to its environmentally harmful impacts and carcinogenic effects to cells of mammals, it has been a great interest in formulating microbial agents to degrade recalcitrant molecules of this type (Wang et al., 2012). In another study, it was reported that *Exiguobacterium sp*. GS1 grew and significantly reduced Cr (VI) concentration in cultures containing 1 - 9% salt. This result indicates that these bacteria can tolerate high salt concentrated environment (Okeke, 2008). Other *Exiguobacterium* stains were also reported to be able to rapidly reduce Cr^{6+} over a wide range of temperature, pH, and salinity. Sa (Alam and Malik, 2008). Stain Exiguobacterium sp. 2 exhibited excellent ability to remove pesticide (Cortés et al., 2006) and stain WK6 was found to have capability to convert arsenate to arsenite (Anderson and Cook, 2004).

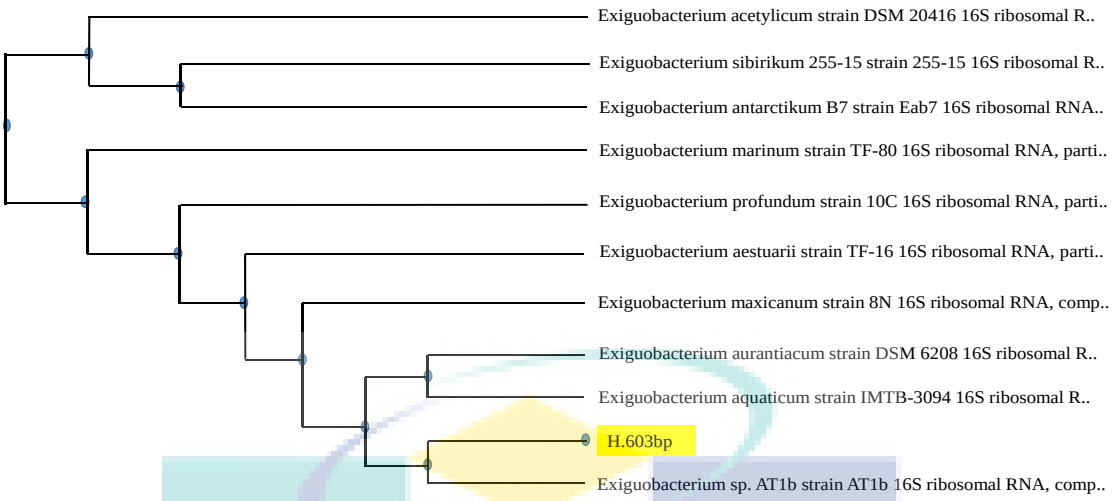


Figure 4.23: Phylogenetic of Bacterial isolate (H) *Exiguobacterium sp*. by NCBI Blast Tree Method

The isolated sample (A) 3 and (B) R were identified using PCR as a *Bacillus anthracis str. Ames.* The phylogenetic of bacteria is presented in Figure 4.24. *Bacillus anthracis* belongs to a gram-positive bacterium which forms endospore and it has capability to infect livestock as well as humans (Poretz, 1979). Together with the protective antigen- a pore-forming unit- virulent stains of *Bacillus anthracis* can cause disease by secreting the lethal factor and edema factor toxins, (Stanley et al., 1960; and Leppla, 1982). The infection spreads to humans mainly through open wound in contact with products which are derived from contaminated animals, when these materials are ingested or inhaled (Brachman, 1984). The presence of these bacteria in river water is mainly from slaughterhouse and livestock surrounding Galing river area.

Bacillus anthracis which is rod in shape, is, 1-1.2µm wide and 3-5µm long. this organism resides in soils throughout the world at mesophilic temperatures (Lal and Tabacchioni, 2009) and can live in both aerobic and anaerobic conditions (facultative anaerobe) in media providing essential nutrients, such as nitrogen and carbon sources (Raines et al., 2006). Its cellular size, morphological features, and formation of spore are identical with *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus thuringiensis* (Raines et al., 2006).

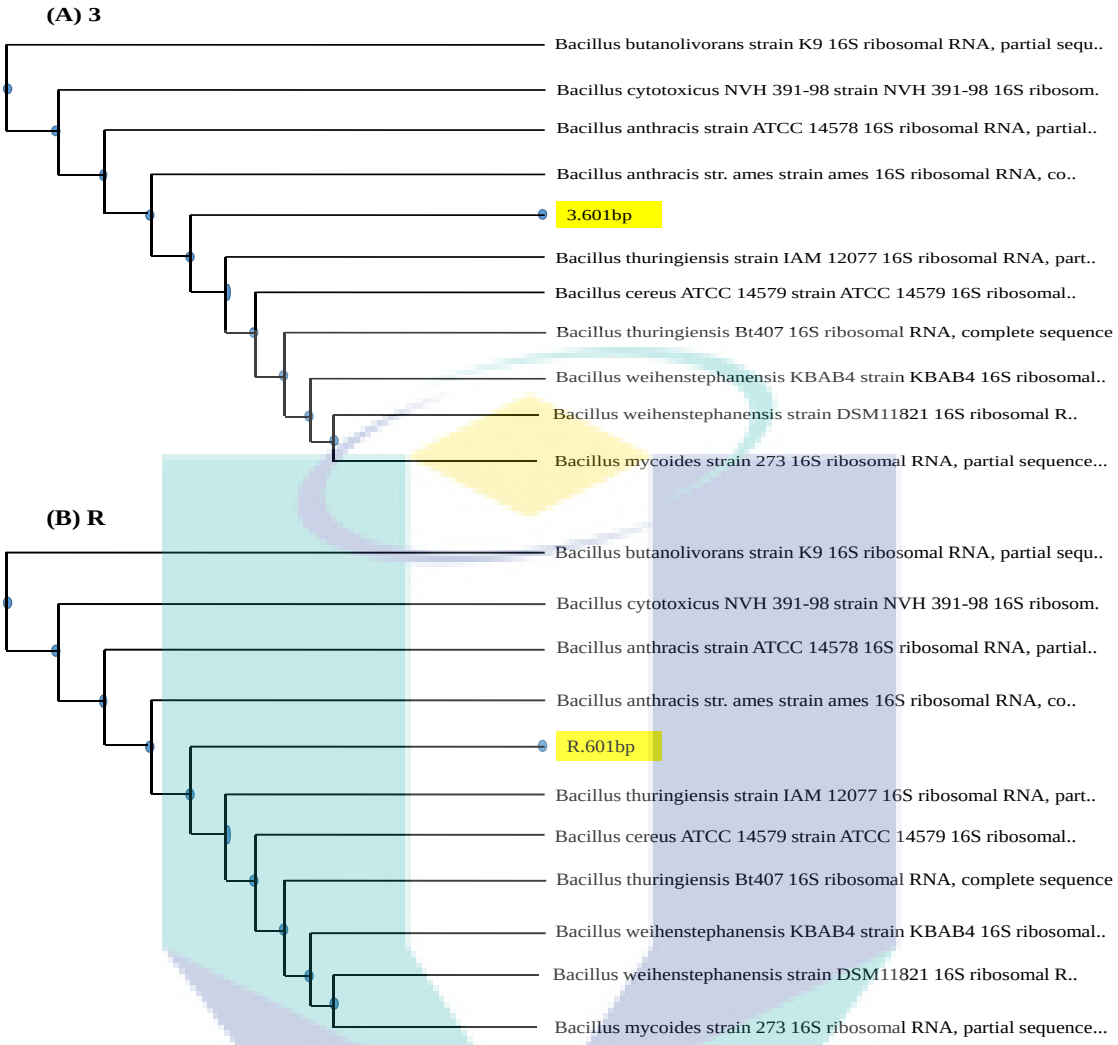


Figure 4.24: Phylogenetic of Bacterial isolate (A) 3 and (B) R *Bacillus anthracis str. Ames* by NCBI Blast Tree Method

Furthermore, genome sequencing is useful for vaccines development. As for *anthrax* disease, one of the important research areas, one of which is the interactions between the cells of host's immune system and the spores will reveal a better understanding. Developing a tool for better detecting spore is also helpful (Liu et al., 2004). These organisms are referred with other names, such as *Bacterium anthracis* and *Bacillus cereus var. anthracis*, but it is commonly known as "*anthrax*" and "*anthrax bacterium*" (Wheeler et al., 2000), These bacteria are also presented in the phylogenetic tree. Another type of *Bacillus sp.* such as a *Bacillus butanolivorans sp.* are used in industrial applications for the remediation of n-butanol which was reported by Kuisiene et al. (2008).

4.11 BIODEGRADATION OF WCO USING ENRICHMENT BACTERIA ISOLATES FROM GALING RIVER WATER IN BATCH PRODUCTION

4.11.1 Determination of Bacteria Growth in Batch Production Using Various Concentrations of Waste Cooking Oil (WCO) to Times Degradation from M4 Sampling Locations of Galing River Water with Optical Density (OD) Analysis

The degradation of WCO using bacterial stain isolated from contaminated water of Galing River was conducted by selective enrichment techniques: Initially, the microorganisms was aimed to WCO degradation which was widely distributed in nature. And was isolated from the aquatic ecosystem of Galing River. The degradation of WCO using bacteria isolated from the M4 (downstream Vistana hotel) was investigated. There were five bacteria identified, namely *Providencia stuartii, Bacillus pimilus, Exiquobacterium sp.,* and *Bacillus antracis.* All isolates of the bacteria were used to degrade the WCO using media concentration and time as well as variations in the number of bacterial isolates. The degradation time (1 to 4 days) vary with concentration of media growing (1 - 4%) at a temperature of incubation conditioned 30 ˚C. The optical density was measured to determine the ability of bacteria to grow and degrade WCO as growing media.

The result of optical density measurement in M4 sampling point as shown in Figure 4.25 reveals that the cell density was initially at low level on the first day. By the time of measurement, then it significantly increased from the day two to the day three of observation. In the following three days, the cell density remained relatively constant, but in the day four, it decreased. The rate of degradation rate marked only a few percent on the first day, but in the following days, it linearly increased. From the growth curve, it can be observed that on the first day, the bacteria were adapting with to the environment which one of the indicators is that they were not able to immediately grow and reproduce. During this period on the first day, the bacteria requires time to grow and reproduce in new culture media. The phase where bacterial metabolism and these was dynamic and rapid formation of new cellular material which occurred on the second day and the third day, the bacteria grew in logarithmic pattern. In the stationary phase, the numbers of bacteria were steadily constant starting from the fourth day.

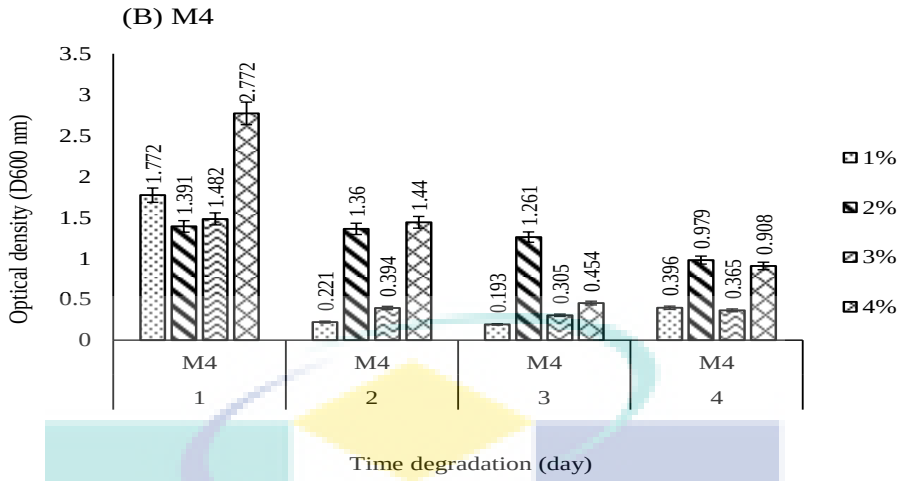


Figure 4.25: Biodegradation rate of waste cooking oil by isolated bacteria. The incubation conditions were 30 ˚C, and the initial WCO concentration was 1-4% (v/v), all data were resulted from four days incubation during the process of four days. The sampling location (B): M4 downstream Vistana hotel

The effect addition to WCO concentration was also investigated. From the Figure 4.25, it shows that the different concentrations of WCO as growing media affect the OD value. The highest value of OD on first day reached 2.77 at 4% concentration of the WCO, but it declined when the concentration of WCO was added up to 4%. The addition of WCO enriched nutrients to the media, thus the bacteria would degrade WCO. This result is in line with treatment at M4 sampling point, increasing the time of degradation could decrease the OD value of bacteria. Meanwhile, increasing WCO concentration up to 2% in the incubation phase resulted in increasing the OD value. Yet, by addition up to the 4% was found to decline the OD value.

4.11.2 Degradation of Waste Cooking Oil (WCO) by Different Concentration of Bacteria from M4 Sampling Locations in Galing River Water with Gravimetric Analysis

A close relationship between variation bacteria from (M4) 50, 75, 100 µl has been reported in the literature elsewhere (Latha and Kalaivani, 2012). The gravimetric analysis of different percentage of bacteria concentration and the percentage of degraded WCO media as shown in the picture Figure 4.26. Bacteria were identified, namely *Providencia stuartii, Bacillus pimilus, Exiquobacterium sp.* and *Bacillus antracis*. All isolates growing in a bacteria liquid medium, were used to degrade the

WCO using media concentration and time as well as variations in the number of bacterial isolates. By varying the growing media from 0.50, 0.75 to 100 µl with the bacteria concentration of 1, 2, 3 and 4 wt. %, from variations bacterial isolates and variation, waste cooking oil in within 4 days of observation, a different curve was obtained. It indicates the influence of providing treatment of bacterial variation and the amount of waste cooking oil.

The effect of different concentration of bacteria (50, 75, 100 µl) from M4 sampling points in degrading WCO of various concentrations were from 1 to 4% as shown in Figure 4.26. The increased concentration from 50 up to 100 µl lead to increase the percentage of degraded WCO. The highest amount of degraded WCO was found at 100 µl concentration of bacteria was 4%. Concentration of growing media indicates a value of 91.39% M4 sampling point. The result is presented in Figure 4.26 (B). The lowest percentage of degraded WCO was shown at bacteria 50 µl with concentration of 1 %. This result is low due to dense bacteria and WCO as a hydrocarbon source in a very limited amounts of growing media that will lead to the competition among the bacteria to obtain nutrient from WCO. Likewise, the variation bacteria 75 µl also gave similar trend in the effect of had percentage of degraded WCO. The increasing percentage of WCO concentration increased the degradation rate, while the increasing concentration of bacteria was observed to increase the percentage of degraded WCO.

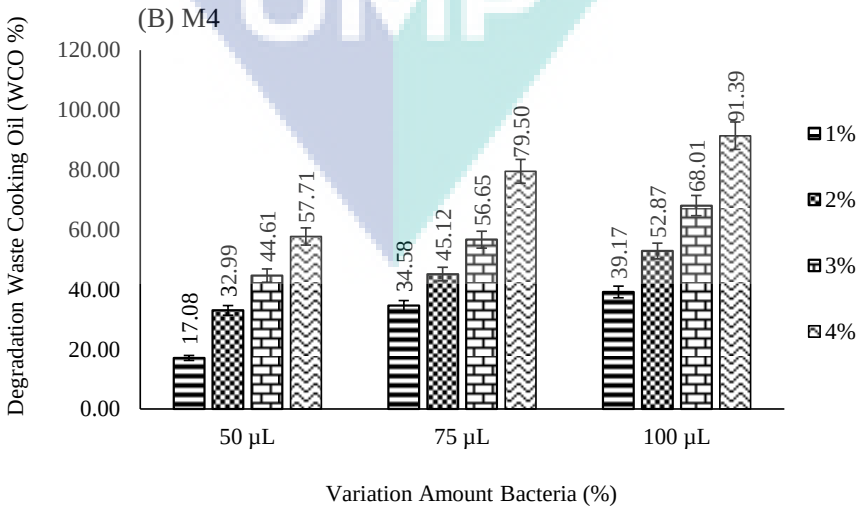


Figure 4.26: Analysis of the effects of variations in 0.50, 75 and 100 µl bacterial to degradation 1-4% waste cooking oil (WCO) from point sample (B): (M4) downstream Vistana hotel in the Galing River

Based on the results in Figure 4.27, it shows that the different number of bacteria to various concentration of WCO from 1 to 4 % has significantly improved the WCO degradation process. Shalaby and Nour (2012), state that bacteria have the ability to grow and reproduce only if the main factors such as the availability of nutrients meet the requirement and an appropriate environment to grow. This result is in accordance with the existing data that in the sample (M4) the number of bacteria varies by as much as 50µl shown the number of bar graphs that WCO has continued to rise. At 50 µl of bacteria, the sample 4% has the highest degradation rate by 57.71 % when compared with 1 % which has the degradation rate only by 17.08 %, and continues to rise 2% and 3%, 39.99% and 44,61 % respectively, on a various concentration of media. The similar trend was observed in the variation 75µl it shows the number of degradation had increased with the increase of addition bacteria, in the fourth variation of media. The highest number of degradation was recorded at 4% with the value 79.50 and 1% variation media 34.58 and continues to rise for 2% degradation rate by 45.12 and 3% degradation rate by 56.65, respectively.

When interpreting the different results from the study, there are also some factors that should be taken into account. As it has previously been mentioned that the location (M4) downstream Galing River is located within the neighboring area with various industrial activities, thus it has a high level of pollution compared with sampling point which is in no contact with a hotel, shopping center, as well as residential areas. According to Dhall et al. (2012), the degree of being contaminated of different locations combined with different source of pollution will result in distinctive degrading bacteria. Based on the discussion above, we can draw conclusion about the ability differences in degrading WCO and also determine the other aspects such as certain type of bacteria recognition, source of contamination which contribute to environmental recovery. Based on the results presented in Figure 4.27, it can be observed in the quantity of bacteria, at concentration of WCO 4 % it gives the most significant improvement in WCO degradation process.

Figure 4.27 reveals that improvement of bacterial degradation capability in various media. However, the rapid changes in growing media concentration has an effect on the bacteria 100 µl, and 4% adding WCO which is shown in the sample (M4)

accounting for 91.39 %. This is much better when compared with the sample bacteria 100 µl which variation WCO 3% which has only 68.01 % rate of degradation. It means that the degradation rate of bacteria in the sample 100 µl variation WCO 2% is better compared with sample 1% of the same concentration of bacterial as 100 µl rates of bacteria 52.87% at 2% variation bacteria and 39.17 % at 1% variation bacteria respectively, (shown in Figure 4.27). Meanwhile, the degradation ability of each sample increases at high of every level variation of bacteria and concentrations of WCO. 4% have a higher degradation rate compared to the variation of 1, 2 and 3%.

However, so far there has been little discussion about the degradation of the WCO bacteria which is to determine the effect of variations of the number of bacteria regarding their biodegradation ability. The degree of WCO degradability is shown based on Figure 4.27. Still, too little attention has been paid to sources of pollution and bacterial types that can break down contaminants by utilizing resources available from bacteria at the site of pollutant sources. Most of the research conducted in Kuantan, Malaysia covered a few parts of regions (Heinaru et al., 2000). Most of the previous researches were merely focused on identifying sources of pollution rather than preventing environment from pollution or solely on recovering the polluted areas. In addition, there are some studies which have found that the surveyed sites had been contaminated by a variety of wastes. So far this method is only applicable for monitoring contaminating waste at certain locations. Several studies have established estimation on bacteria that utilize more effectively pollution sources and have cheaper costs (Stephen et al., 2005). Still, there is insufficient data to compare waste degradation ability in isolation from contaminated sources with different concentration of bacteria as shown in Figure 4.27.

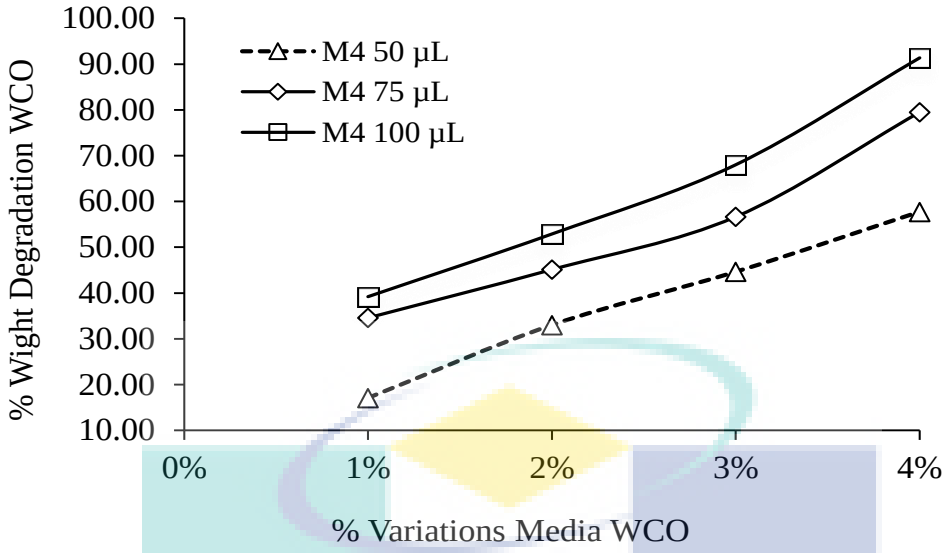


Figure 4.27: Analysis of the effects of variations in 0.50, 0.75 and 100µl bacterial to % degradation waste cooking oil from (WCO) point sample (M4) downstream vistana hotel the Galing River area

Previous literatures written by some experts, such as Laukova et al. (2002), reported that the bacterium has four phases in its life, namely the first is lag phase or initial period. The second phase is exponential phase, subsequently stationery and last phase is death. According Kummerer, 2004; Malik and Ahmed. (2002), contamination at different locations as well as different sources of pollution will have different degrading bacteria. It is also related to the variation of bacterial growth media and the amount of bacteria that is used for waste degradation. Literature also revealed that many other factors can contribute to this condition, such as effect of treatment, media, environmental conditions, type and amount of bacterial colony.

4.12 CHARACTERIZATION OF WASTE COOKING OIL DEGRADED WITH BACTERIA ISOLATES USING FT-IR ANALYSIS

Characterization of waste cooking oil in advanced research aims to determine the peaks of the waste cooking oil after the treatments using bacteria, the peaks mentioned are ability of bacteria in the reduction of chemical substances that are naturally reduced, thus requiring the degrading microorganisms to be able to break down in the environment polluted. Testing bacteria to degrade the waste cooking oil using FTIR analysis can be seen in the Figure 4.28. Gravimetric method uses the

difference analysis by FTIR analysis, namely: gravimetric is to determine the amount of oil that is degraded while FTIR analysis is to determine the peaks waste cooking oil in reduction to be easily biodegradable in the environment. Characterization WCO by FTIR analysis use the isolation of bacteria originating from the M4, ie *Providencia stuartii, Bacillus pumilus, Exiguobacterium sp.,* and *Bacillus anthracis Ames* in a single test.

In order to determine the effect of the microbial addition in the biodegradation WCO, which is *Providencia stuartii*, *Bacillus pumilus*, *Exiguobacterium* sp., and *Bacillus anthracis Ames*, the experiments with varying microbial concentration (50, 75 and 100 µl) and various WCO (1-4%) amount were conducted under the optimal reaction conditions. The trend from the Figure 4.27 was shown by the addition of microbial in the batch production, new peak at several wavenumber was observed, and it means the microbial has the capability to degrade the WCO. From the Figure 4.28, the new peaks were observed at 721 and 869 cm-1 showed the presence of aromatic compound due to benzene ring. Band at 1646 cm-1 is attributed the olefins group (C=C), peaks of 2028 are attributed the C=O group, peak at 2324 cm-1 is attributed of the CO oxidation products such as physisorbed CO2 peaks of 2727 cm-1 due to C-H deformation, conjugated cyclic and CHO are also observed. It can be assumed that the C=C aromatic bonds presence were after the addition of bacteria and converted as aliphatic C=C stretching bond to aromatic.

FTIR provides a quick and accurate way to evaluating structural changes of waste cooking oil (WCO) subjected to degradation by bacteria (Chuanhao, 1999). The effect of biodegradation WCO by bacteria isolated from Galling river water namely M4 (downstream Vistana hotel) was observed using FTIR spectrophotometer. Infrared of the WCO and residual oil reveals several absorption peaks and shifts in the maximum wavelength of transmittance which confirms the modification of the original oil by microbial treatment (Figure 4.28). The IR spectra of WCO (origin) in all. Figure 4.28 shows the characteristics bands at 3478, 2927, and 2675 cm-1 is attributed to the symmetric stretching vibration of the aliphatic CH2 and CH3 group; 1750 cm-1 band is attributed to C=C stretch in aromatic nuclei and 1465 cm-1 assigned to the vibrations of deformation δ(C H); band at 1380 cm-1 assigned to deformation vibration

of methylene group; 1162 cm-1 is characteristic to vibration of C=O bonds, and 1106 cm-1 are assigned to vibration of C=C bonds of carbohydrate chain from oil composition. All bands in WCO are matched with characteristic spectra IR for cooking oil (Biswal and Bhadouriya, 2012, Poiana et al.,2012).

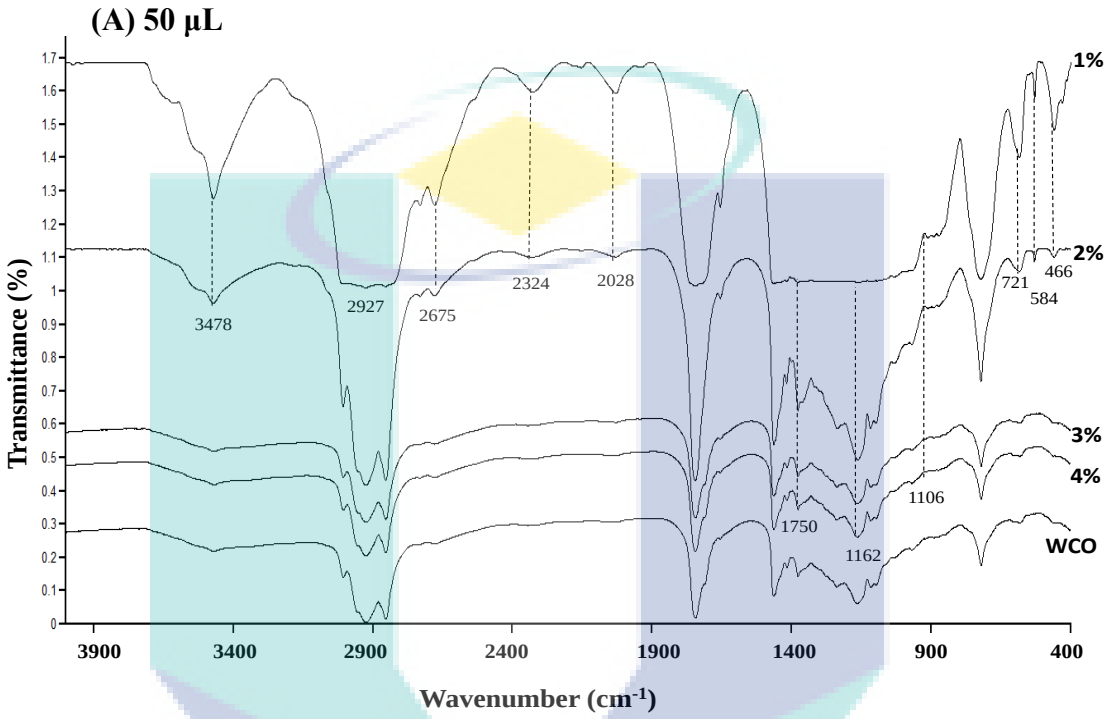


Figure 4.28: FTIR spectrum of different concentrations of waste palm cooking; (A) 50, (B) 75 and (C) 100 µl, with varied addition (1-4%) of bacteria in point M4 downstream vistana hotel from Galing River

The FTIR spectra of the various bacteria loading (50, 75 and 100 µl) in the Figure 4.28 shows a similar trend. In contrast, the addition of various WCO (1-4%) has shown a different depict FTIR spectra, with decrease in the amount of oil content showing the changes of the FTIR spectra. It can be concluded the microbial from M4 (*Providencia stuartii, Bacillus pumilus, Exiguobacterium* sp., and *Bacillus anthracis. Ames*) has a capability in the degradation of hydrocarbon (oil). In line with (Maliji et al., (2013) reported *Bacillus* sp. has shown a maximum diesel oil degradation (82.41% and 81.56% of aliphatic and aromatic hydrocarbons) after 2 days incubation. Furthermore *Providencia* sp demonstrates effectiveness in diesel oil degradation (Ganesh & Lin 2009).

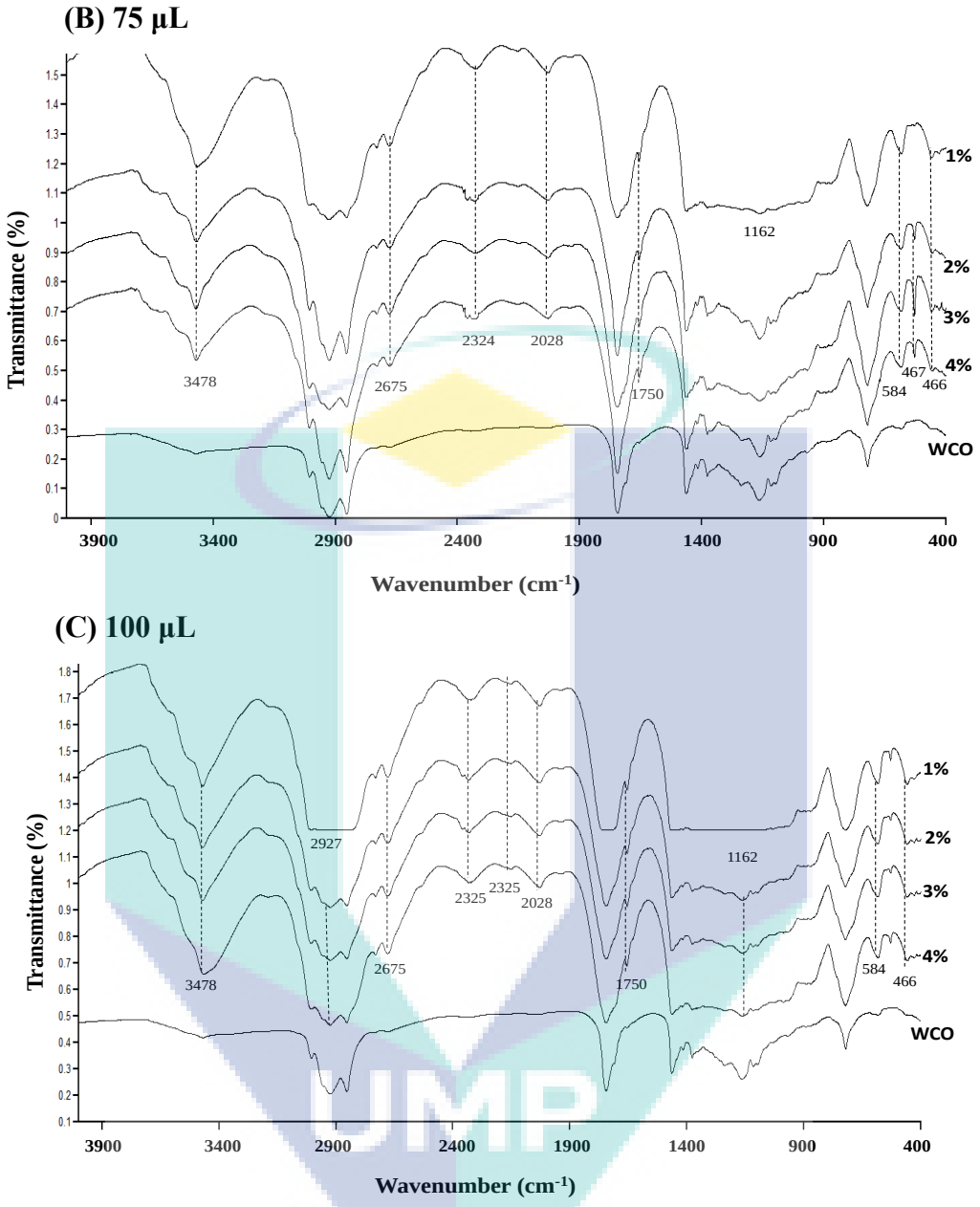


Figure 4.28: Countinued

This work contributes in solving problems with the knowledge of river water being contaminated. By various wastes the results of the study provide an overview of data analysis and provide a discussion of which is supported by a variety of sources. This study consisted of four sampling locations along the Galing River of the industrial area Semambu. The parameters analysis of water quality standard, namely the Physical, Chemical and biological, including pH, temperature, DO, BOD, COD, SS, and AN, and then of the isolation bacteria from water samples, using the agar plate, and to do

screening using gram strain of bacteria obtained names; *Pseudomonas* sp., *Bacillus* sp, *Staphylococcus* sp., *Escherichia coli*, *Salmonella* sp., *Shigella* sp., *Providencia* sp., and *Clostridium*, bacteria the successfully in isolation breeding be composed a very good growth, these is measured CFU and optical density. Bacteria are grown well in degrading waste using Phenol Red lipolytic test, Rhodamine B, MacConkey Agar, and Rapid One bacteria are obtained; *Acinetobacter calcoaceticus*, *Leminorella grimontii* (EG 57), *Shigella* sp., *Providencia stuartii*, and *Moellerella wisconsensis*. The next analysis is to use your identification of bacteria-Specific PCR to obtain names are the names of *Providencia stuartii* obtained bacteria, *Bacillus pimilus*, *Exiquobacterium* sp., and *Bacillus antracis*. Names of bacteria that have the ability to degrade the waste is then tested in a laboratory scale test for the waste cooking oil discusses the degradation of FT-IR and Gravimetric analysis. Chapter 4 results and discussion, the conclusions and suggestions are on the next chapter 5.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

Based on the experiments, there are some research outcomes which can be summarized as listed below.

The characteristics of biological, chemical and the physical parameter of Galing river water were obtained. Based on research conducted using the formula WQI method of calculating the value of pH, DO, BOD, COD, SS, and AN, it can be concluded that the water quality was observed by sampling four locations, namely: M1 Street industrial Semambu included in class IV, M2 Drain Lorong setali included in class III, and M3 Drain Street Galing included in class IV, while the most pollutants from the fourth location of namely M4 Downstream sample Vistana Hotel with value 32.61 WQI. Galing river water also contains the most pollutants is oil and grease which were at the location of the M4 with a value of 14 mg / L, while the cleanest currently on M1 with a value of 2 mg / L, while M2 and M3 respectively 4 mg / L and 7 mg / L based on an analysis method by (APHA) *American public health association*.

The isolation and identification of potential lipolytic bacteria originating from the location of the M4 downstream Vistana hotel at the Galing River water, was obtained using several ways, namely: Identification by using the agar plate. screening process was performed using gram strain, Measurement of the optical density and CFU had obtained 8 groups, *Pseudomonas* sp., *Bacillus* sp., *Staphylococcus* sp., *Escherichia coli*, *Salmonella* sp., *Shigella* sp., *Providencia* sp., and *Clostridium*. Then the bacteria tested using Phenol Red, Rhodamine B, MacConkey agar, and rapid one test. The tests

were chosen as a biochemical test for initial identification, as it is a simple and fast method to identify bacteria. The test results indicated a possibility of utilizing the stains, namely; *Acinetobacter calcoaceticus, Leminorella grimontii* (EG 57), *Shigella* sp., *Providencia stuartii*, and *Moellerella wisconsensis*. The next analysis was carried out to identify DNA and PCR of specific bacteria. This test obtained data: such as *Providencia stuartii, Bacillus pimilus, Exiquobacterium* sp., and *Bacillus antracis*.

The measurement of lipolytic bacteria was capable of degrading the waste cooking oil sources (WCO) in batch production based on data observed optical density and gravimetric analysis, and then seen by the degradation of WCO using FTIR method. Based on the analysis it was found, namely: The maximum OD value of 2.77 at a concentration of 4% on the first day. Whereas gravimetric analysis had a maximum value of 91.39% degradation in 100 ml variation bacteria and 4% WCO, respectively 79.50% degradation in 75 µl variation bacteria and 57.71% in 50 ml bacterial variation. It is grounded in a variety of media (50, 75 and 100 µl) of bacteria and 1-4% WCO. While the minimum value of the variation of 50 µl bacteria and 1% WCO degradation 17:08%, respectively, than 34.58% degradation in 75 µl bacteria variations. And 39.17% degradation in the variation of bacteria 100 ml WCO. This trend continues to increase from 1-4% with variation bacterial of (50, 75 and 100 µl). While the degradation of WCO using FTIR was obtained, the new peaks were observed at 721 and 869 cm^{-1} and was shown in the presence of aromatic compound due to a benzene ring. The peak at 2324 cm^{-1} is attributed of the CO oxidation products such as physisorbed CO_2 peaks of 2727 cm^{-1} due to C-H deformation, conjugated cyclic and CHO are also observed. It can be assumed that the C=C aromatic bonds presence after the addition of bacteria and converted as aliphatic C=C stretching bond to aromatic. FTIR provides a quick and accurate way to evaluating structural changes of waste cooking oil (WCO) subjected to degradation with bacteria. Absorption peaks and shifts in the maximum wavelength of transmittance which confirms the modification of the original oil by microbial treatment.

5.2 RECOMMENDATION

Though the research had successfully investigated a series of experiments on studies on Galing river water: isolation, identification of lipolytic bacteria and biodegradation of water quality in Kuantan, Pahang, a number of recommendations are proposed to enhance the whole research as listed below:

1. It is required to be more active in monitoring river for the one-year system. Emphasis should be taken with the effluent standard discharge into the river as a good water management indication. Improvement of a policy for the preservation of the river system, which will be responsible for controlling the pollution in the river should be executed.

2. An online monitoring system is to be installed by the authority to monitor the pollution discharge by all the industrial wastewater stakeholders.

3. For further research, more work are needed in isolation. For example, the use of different media should be tested, by adding or removing some compounds to verify what bacteria which is able to survive or be dead in each case.

4. The identification should be supplemented in terms of some basic conventional biochemical test, in order to complement the result from RapID ONE. In the same way, a thorough and complete Gram staining and biochemical test for all the eighteen bacterial isolates should be conducted to investigate the potential lipolytic of each and every bacterium found in this research.

REFERENCES

Abdel-Raouf, N., Al-Homaidan, A.A. and Ibraheem, I.B.M. 2012. Review Microalgae and wastewater treatment. *Saudi Journal of Biological Sciences*. **19,**: 257–275.

Abdo, M.H., Sabae, S.Z., Haroon, B.M., Refaat, B.M. and Mohammed, A. 2010. Physico-Chemical Characteristics, Microbial Assessment and Antibiotic Susceptibility of Phatogenic Bacteria of Ismailia Canal Water, River Nile, Egypt. *Journal of American Science,*. **6**((5)): 234–250.

Abul Kashem, M.D., Singh, B.R. and Kawai, S. 2007. Mobility and distribution of cadmium, nickel and zinc in contaminated soil profiles from Bangladesh. *Nutrient Cycling in Agroecosystems,*. **Vol. 77,**: pp: 187–198.

Adley, C.C. and Saieb, F.M. 2005. Comparison of bioMérieux API 20NE and Remel RapID NF Plus, identification systems of type strains of Ralstonia pickettii. *Letters in applied microbiology*. **41**(2): 136–40.

Aghapour, A.A., Moussavi, G. and Yaghmaeian, K. 2013. Biological degradation of catechol in wastewater using the sequencing continuous-inflow reactor (SCR). *Journal of Environmental Health Science & Engineering*. **11:3**.

Ahmad, A., Mushrifah, I. and Lim, E.C. 2008. Seasonal Influence on Water Quality and Heavy Metals Concentration in Tasik Chini , Peninsular Malaysia. : 300–303.

Ai, T.T.T., C.M.Kao, Surampalli, Y., R. and Chien, H.Y. 2009. Enhanced Bioremediation of Fuel-Oil Contaminated Soils: Laboratory Feasibility Study. *Journal of Environmental Engineering,*. **Vol. 135,**: No. 9,.

AL, K., Jones RG and Reynard NS. 2006. RCM rainfall for UK flood frequency estimation. II. Climate change results. *Journal Hydrol,*. **Vol. 318,**: pp: 163–172.

Alam, M.J.B., Islam, M.R., Muyen, Z., Mamun, M. and Islam, S. 2007. Water quality parameter along rivers. *Int. J. Environ. Sci. Tech.,*. **4**: (1): 159–167.

Alam MZ. and Malik A. 2008. Chromate resistance, transport and bioreduction by Exiguobacterium sp. ZM-2 isolated from agricultural soil irrigated with tannery effluent. *Journal of basic microbiology.*. **48(5):** 416–20.

Alfred, E.B. 2005. Benson's microbiological applications: Laboratory manual in general microbiology. 9th ed. *USA: Electronic Publishing Services Inc.*

Allan, I.J., Vrana, B., Greenwood, R., Mills, G.A., Knutsson, J. and Holmberg, A. 2006. Strategic monitoring for the European water framework directive. *TrAC, Trends in Analytical Chemistry,*. **Vol. 25.**: pp:704–715.

Alloway, B.J. and Ayres, A.K. 1997. Chemical Principles of Environmental Pollution, second ed. : p. 214.

Al-Masri, M.S., Aba, A., Khalil, H. and AL-Hares, Z. 2002. Sedimentation rates and pollution history of a dried lake: ALOteibeh lake. *Science of the Total Environment,*. **Vol. 293,**: pp: 177–189.

Ammar, E., Nasri, M. and K Medhioub 2005. Isolation of phenol degrading Enterobacteria from the wastewater of olive oil extraction process. *World Journal of Microbiology and Biotechnology,*. **21**((3)): 253–259.

Anderson CR. and Cook GM. 2004. Isolation and characterization of arsenate-reducing bacteria from arsenic-contaminated sites in New Zealand. *Current microbiology.*. **48(5):** 341–7.

Ankley, G.T., Lodge, K., Call, D.J., Balcer, M.D., Brooke, L.T. and Cook, P.M. 1992. Integrated assessment of contaminated sediments in the lower Fox River and Green Bay Wisconsin. *Ecotoxicology and Environmental Safety,*. **Vol. 23**((1),): pp: 46–63.

Arku, A.Y., Musa, S.M. and Mofoke, A.L.E. 2012. CHARACTERIZATION AND STANDARDIZATION OF CRUDE MORINGA OLEIFERA SEEDS FOR WASTEWATER TREATMENT. *Journal of Applied Phytotechnology in Environmental Sanitation*. **1**(2): 67–74.

Asamudo, N.U., Daba, A.S. and and O. U. Ezeronye 2005. “Bioremediation of textile effluent using Phanerochaete chrysosporium,.” *African Journal of Biotechnology,*. **vol. 4,**(no. 13,): pp. 1548–1553,.

Ash, C., Priest, F.G. and Collins., M.D. 1993. Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus Paenibacillus. *Antonie Van Leeuwenhoek.*. **64**((3-4):): 253–260.

Atlas, R.M. 1995. Petroleum Biodegradation and Oil Spill Bioremediation. *Marine Pollution Bulletin*. **31,**: 178–182.

Bahig, A.E., Aly, E.A., Khaled, A.A. and Amel, K.A. 2008. Isolation , characterization and application of bacterial population from agricultural soil at Sohag Province , Egypt. . **4**(2): 42–50.

Bahrudin, H. Bin 2008. *Bioremediation of oil contaminated wastewater using mixed culture*. University Malaysia Pahang.

Bako., S.P., Chukwunonso., D. and Adamu., A. k. 2008. Bio-Remediation of Refinery Effluents By Stains of Pseodomonas Aerugenosa and Penicillium Janthinellum. *applied Ecology Environmental Research*. **6**((3)): 49–60.

Ban-Weiss A, G., Bala, G., Cao, L., Julia, P. and Caldeira, K. 2011. Climate forcing and response to idealized changes in surface latent and sensible heat. *ENVIRONMENTALRESEARCHLETTERS*. **6**((8pp)): 034032.

Bao, J.L.K. 2010. *Water quality study and its relationship with high tide and low tide at kuantan river*. Universiti Malaysia Pahang.

Bartolomeo, D.A., Poletti, L., Sanchini, G., Sebastiani, B. and Morozzi, G. 2004. Relationship among parameters of lake polluted sediments evaluated by multivariate statistical analysis. *Chemosphere,*. **Vol. 55,**: pp: 1323–1329.

Bartram, J. and Ballance, R. 1996. *Water quality monitoring . a practical guide to the design and implementation of freshwater quality studies and monitoring programmes* 11th ed. J. B. and R. Ballance, ed. London: United Nation Environment Programe.

Beauchamp., C.J., Simao-Beaunoir., A.-M., Beaulieu., C. and Franc-ois-Philippe Chalifour. 2006. Confirmation ofE. coliamong other thermotolerant coliform bacteria in paper mill effluents, wood chips screening rejects and paper sludges. *WATER RESEARCH*. **40 (2006)**: 2452– 2462.

Begum, A., HariKrishna, S. and Khan, I. 2009. Analysis of Heavy metals in Water, Sediments and Fish samples of Madivala Lakes of Bangalore, Karnataka. *Int. J. of ChemTech Res.*. **Vol. 1(2),**: pp: 245–249.

Bengtsson, G., Nordstorm, S. and Rundgren, S. 1981. Population density and tissue metal concentration of lumbricids in forest soils near a brass mill. *Environmental Pollution*. **30,**: pp: 87–108.

Berberoglu, S. and Akin, A. 2009. Assessing different remote sensing techniques to detect land use/cover changes in the eastern Mediterranean. *International Journal of Applied Earth Observation and Geoinformation*. **11**: 46–53.

Berg ., Kiibus M. and Kautsky N. 2001. Heavy metals in tropical Lake Kariba, Zimbabwe. *Journal Wat Air Soil Pollut.*. **Vol. 82.**: pp: 1–6.

Bergey, D.H., Holt, J.F., Krieg, N.R. and Sneath, H.A. 1994. Bergey's Manual of Determinative Bacteriology (9th ed.). *Lippincott Williams & Wilkins.*

Botalova, O. and Schwarzbauer, J. 2011. Geochemical characterization of organic pollutants in effluents discharged from various industrial sources to riverine systems. *Water Air Soil Pollut.*

Brachman, P.S. 1984. Anthrax, p. 826-830. In K. S. Warren and A. F. Mahmoud (ed.), Tropical and geographic medicine.McGraw-Hill, New York, NY.

Bragg, R., J., Prince, R.C., Harner, E.J. and Atlas, R.M. 1994. Effectiveness of bioremediation for the Exxon Valdez oil spill. *Nature*. **368,**: 413–418.

Brown JN and Peake, B. 2006. Sources of heavy metals and polycyclic aromatic hydrocarbons in urban stormwater runoff. *Sci Tot Environ*. **359**: 145–155.

Buccolieri A., Buccolieri G, Cardellicchio N, Atti A.D, Leo., A.D. and Antonella M. 2006. Heavy metals in marine sediments of Taranto Gulf (Lonian Sea, Southern Italy). *Marine Chemistry.*. **Vol. 99,**: pp: 227–235.

Burton E., Phillips I. and Hawker D. 2004. Trace metals and nutrients in bottom sediments of the southport Broadwater, Australia. *Mar Pollut Bull.*. **Vol. 48.**: pp:378–384.

Buthelezi Simphiwe P. 2008. *Application of bacterial bioflocculants for wastewater and river water treatment.* University of KwaZulu-Natal, Durban,.

Capuccino, J.G. and Natalie S. 2000. Microbiology A Laboratory Manual. Benjamin/Cummings Publishing Company Inc., Menis Park, California.

Chapman PM., McDonald BG. and Lawrence GS. 2002. Weight-of-evidence issues andframeworks for sediment quality (and other) assessments. *Hum Ecol Risk Assess.*. **Vol. 8,**: pp: 1489–1515.

Chowdhuri, A., Iqbal, A., Giasuddin, M. and Bhuiyan., A.A. 2011. Study on Isolation and Identification of Salmonella and Escherichia coli from Different Poultry Feeds of Savar Region of Dhaka, Bangladesh. *Journal of Scientific Research, J. Sci. Res.*. **3**((2)): 403–411 (2011).

Chowdhury, a., Iqbal, a., Uddin, M.G. and Uddin, M. 2011. Study on Isolation and Identification of *Salmonella* and *Escherichia coli* from Different Poultry Feeds of Savar Region of Dhaka, Bangladesh. *Journal of Scientific Research*. **3**(2).

Christian R., Blaese, Armstrong. and B., J. 2008. Lipolytic Bacteria in the Ottawa River. *American Society for Microbiology,*. **26**((5):): 733–740.

Christine M. Hegedus., C.F.S. et al. 2008. Decreased Urinary Beta-Defensin-1 Expression as a Biomarker of Response to Arsenic. *TOXICOLOGICAL SCIENCES106*. **(1),**: 74–82.

Christopher, K. and Bruno, E. 2003. Identification of Bacterial Species. In Alberta, CANADA: Department of Biological Sciences, University of Alberta Edmonton,.

Chuanhao, W. 1999. Analysis of aromatic compounds in water by ion mobility spectrometry ... *ProQuest Dissertations and Theses*: 48106–1346.

Coker, W.B., Kettles, I.M. and Shilts, W.W. 1995. Comparison of mercury concentrations in modern lake sediments and glacial drift in the Canadian Shield in the region of Ottawa/Kingston to Georgian Bay, Ontaria, Canada. *Water, Air and Soil Pollution,*. **Vol. 80,**: pp: 1025–1029.

Cortés A.L, Schumann P., Pukall R. and Stackebrandt E. 2006. Exiguobacterium mexicanum sp. nov. and Exiguobacterium artemiae sp. nov., isolated from the brine shrimp Artemia franciscana. *Systematic and applied microbiology.*. **29(3):** 183–90.

Corynebacterium, A., Staphylococci, E.N.N., Yeast, S., Savings, T., Savings, L. and Database, E. 2009. RapID ™ Systems for Identification of : RapID ™ Systems and Accessory Products Quick Reference Guide.

Cserháti, T., Forgács, E. and Oros, G. 2002. Biological activity and environmental impact of anionic surfactants. *Environment International*. **28**(5): 337–348.

Daneshmand S., ., Huat, B.B.. and Moayedi, H., and Ali, T.A.M. 2011. Study on Water Quality Parameters of Linggi and Melaka Rivers Catchments in Malaysia. *Engineering Journal,*. **Vol 15,**: No 4.

Daniel, J. 2011. *Nebraska public water supply program summary report 2010. annual report. nebraska department of health and human services*.

Dassenakis, M., Scoullus, M., Foufa, E., Kraskopoulou, E., Pavlidou, A. and Kloukiniotou, M. 1998. Effects of multiple sources pollution on a small Mediterranean river. *Appl. Geochem*. **Vol. 13**((2).): pp: 197–211.

Dhall., P., Kumar., R. and Kumar, A. 2012. Biodegradation of Sewage Wastewater Using Autochthonous Bacteria. *The Scientific Word Journal*. **2012**: 8.

Dhanve RS., DC., K., Phugare SS. and Jadhav JP. 2009. Coordinate action of exiguobacterial oxidoreductive enzymes in biodegradation of reactive yellow 84A dye. *Biodegradation*. **20:** 245–255.

Dhouib, A., Ellouz, M., Aloui, F. and Sayadi, S. 2006. Effect of bioaugmentation of activated sludge with white-rot fungi on olive mill wastewater detoxification. *Letters in Applied Microbiology,*. **42**((4)): 405–411.

Diaz, E. 2004. Bacteria Degradation of Aromatic pollutants: a paradigm of metabolic versatility. *International Microbiology*. **7:** 173–180.

Diez, M.C. 2010. Biological Aspects Involved in the Degradation of Organic Pollutants. *Journal Soil Science Plat Natural*. **10**(3): 244–267.

DOE 2011. Department of Environment (DOE) Malaysia Environmental Quality Report 2011. : p. 24 & 84 – 85. Misas Advertising Sdn. Bhd.

DOE 1974. *Department of environment, report by central unit on environmental pollution*, Third Edit. by R.M. Harisson., ed. Cambridge CB4 0WF, UK.: Published by The Royal Society of Chemistry, Thomas Graham House, Science Park, Milton RoadPublished by The Royal Society of Chemistry, Thomas Graham House, Science Park, Milton Road.

Domde., P., A., K. and Purohit J.H. 2007. Impact of Bioaugmentation with a Consortium of Bacteria on the Remediation of Wastewater-Containing Hydrocarbons. *Journal Env. Sci. Pollut.,*. **14 (1):** 7 – 11.

Edema, N. 2006. Effects of Crude Oil Contaminated Water on the Environment.

EPA 1990. U.S. Enviromental Protection Agency Interim Report, Oil Spill Bioremediation Project. U.S. Environmental Protection Agency,. *Office of Research and Development, Washington.*: 143–148.

Erguder, T.H., Guven, E. and Demirer, G.N. 2000. Anaerobic treatment of olive mill wastewaters in batch reactors. *Process Biochemistry,*. **36**((3)): 243–248.

Ettayebi, K., Errachidi, F., Jamai, L., Tahri-Jouti, A.M., Sendide, K. and Ettayebi, M. 2003. Biodegradation of polyphenols with immobilized Candida tropicalis under metabolic induction. *FEMS Microbiology Letters,*. **223**((2)): 215–219.

Fahmi, M., Nasir, M., Samsudin, M.S., Mohamad, I., Roshide, M., Awaluddin, A., Mansor, M.A., Juahir, H. and Ramli, N. 2011. River Water Quality Modeling Using Combined Principle Component Analysis (PCA) and Multiple Linear Regressions (MLR): A Case Study at Klang River , Malaysia. . **14**: 73–82.

Fakoussa, R. and Hofrichter, M. 1999. Biotechnology and microbiology of coal degradation. *Applied microbiology and biotechnology*. **52**(1): 25–40.

Field, K.G. and Samadpour, M. 2007. Fecal source tracking, the indicator paradigm, and managing water quality. *Water research*. **41**(16): 3517–38.

Focazio, M.J., Kolpin, D.W., Barnes, K.K., Furlong, E.T., Meyer, M.T. and Zaugg, S.D. 2008. A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States—II untreated drinking water sources. *The Science of the Total Environment,*. **Vol. 402,**: pp: 201–216.

Ganesh, A. and Lin, J. 2009. Diesel degradation and biosurfactant production by Gram-positive isolates. *African Journal of Biotechnology*. **8**(21): 5847–5854.

Gibson, N.J. 2006. The use of real-time PCR methods in DNA sequence variation analysis. *Clinica chimica acta; international journal of clinical chemistry*. **363**(1-2): 32–47.

Gonzalez-Macias, I, S., Liuch-Cota DB, M.-R.L. and S, H.-V. 2011. Distribution, enrichment and accumulation of heavy metals in coastal sediments of Salina Cruz Bay, Mexico. *Environ Monit Assess,*. **118.**: pp.211–230.

Gordon, R. 1994. Bioremediation and its Application to Exxon Valdez Oil Spill in Alaska. *Journal of Scientific Research,*. **2**.

Hamid H. and Baki, A.M. 2011. Sewage Treatment Trends in Malaysia Series 3. *THE EINGENIEUR*: 46–53.

Hamme, J.D. Van, Singh, A. and Ward, O.P. 2003. Recent Advances in Petroleum Microbiology. *MICROBIOLOGY AND MOLECULARBIOLOGY REVIEWS, Dec. 2003,*. **67(4):** p. 503–549.

Hamza, U.D., Mohammed, I.A. and Sale, A. 2012. Potentials of Bacterial Isolates in Bioremediation of Petroleum Refinery Wastewater. *Journal of Applied Hytotechnology in Environmental Sanitation*. **1**(3): 131–138.

Hamza, U.D., Mohammed, I.A. and Sale, A. 2012. Potentials of Bacterial Isolates in Bioremediation of Petrolium Refinery Wastewater. *Journal of Applied Phytotechnology in Environmental Sanitation*. **1**(3): 131–138.

Hamzah, U.D., Mohammed, I.A. and Sale, a. A. 2012. Potential of Bacteria Isolates in Bioremediation of Petrolium Refinery Wastewater. *Journal of A plied Hytotechnology in Environmental Sanitation,*: 131–138.

Hang, X., Wang, H., Zhou, J., Du, C. and Chen, X. 2009. Characteristic and accumulation of heavy metals in sediments originated from an electroplating plant. *Journal of hazardous Material,*. **Vol. 163.**: pp: 922–930.

Hawkins, A. 2001. Bioremediation of Water Areas Due to Oil Spills.

He, G., Lu, Y., Ma, H. and Wang, X. 2007. Multi-indicator assessment of water environment in government environmental auditing. *Journal of environmental sciences (China)*. **19**(4): 494–501.

Heinaru, E., Truu, J., Stotmeister, U. and Heinaru, A. 2000. Three types of phenol and p-cresol catabolism in phenoland p-cresol-degrading bacteria isolated from river water continously polluted with phenolic compounds. *FEMS Microbiology Ecology*. **31:** 195–205.

Heller, G., Adomas, A., Li, G., Osborne, J., Vanzyl, L., Sederoff, R., Finlay, R.D., Stenlid, J. and Asiegnu, F.O. 2008. Transcriptional analysis of Pinus sylvestris roots challenged with the ectomycorrhizal fungus Laccaria bicolor. *BMC Plant Biol.*. **25:** 8–19.

Hoff, R.Z. 1993. Bioremediation: an overview of its development and use for oil spill cleanup. *Marine Pollution Bulletin*. **29,**: 476–481.

Hoorfar, J., Cook, N., Malorny, B., Wagner, M., De Medici, D., Abdulmawjood, A. and Fach, P. 2003. Making internal amplification control mandatory for diagnostic PCR. *J Clin Microbiol.*. **41:** 5835–5838.

Huo, Z., Yang, X., Raza, W., Huang, Q., Xu, Y. and Shen., Q. 2010. Investigation of factors influencing spore germination of Paenibacillus polymyxa ACCC10252 and SQR-21. *Applied Microbiology and Biotechnology.*. **87**((2):): 527–536.

Irwin, P. 1996. To clean up environmental spill, know your medium. *Electrical World*: 37–40.

Ismail, A. binti 2008. *Isolation, characterization and identification or microorganisms from soil contaminated with pesticide*. University Malaysia Pahang.

Isola K.T.M., Eniola K.I.T., Olayemi A.B. and Igunnugbemi O.O. 2008. Response of Resident Bacteria of a Crude Oil-Polluted River to Diesel Oil. *American-Eurasian Journal of Agronomy*. **1**((1):): 06–09.

Jaak, T., Ene, T., Eeva, H., Ulrich, S., Helmuth, W. and Ain, H. 1999. Comparison of API 20NE and Biolog GN identification systems assessed by techniques of multivariate analyses. *Journal of Microbiological Methods.*. **36:** 193–201.

Jacobson., R., Klang., J., Sinden., C., Regan., C. and Munir., H. 2008. *Dissolved oxygen tmdl protocols and submittal requirements*.

Jaffe, R.I., Lane, J.D. and Bates, C.W. 2001. Real-time identification of Pseudomonas aeruginosa direct from clinical samples using a rapid extraction method and polymerase chain reaction (PCR). *J. Clin. Lab. Anal.*. **15:** 131–137.

Jamalludeen., N., She., Y.M., Lingohr., E.J. and Griffiths., a. M. 2009. Isolation and Characterization of Virulent Bacteriophages againts Escherichia coli Serogroups O1. O2, and O78. *Poulutry Science Association Inc.*: 1694–1702.

Jio., N., Azam., F. and Sanders., S. 2011. *Microbial carbon pump in the ocean*. UNITED STATES, America: Science AAAS.

Kafilzadeh., Farshid., Farhangdoost., M. sadegh., Tahery. and Yaghaboob 2010. Isolation and Identification of phenol degrading bacteria From lake Parishan and Their Growt Kinetic Assy. *African Journal or Biotechnology*: pp. 6721–6726.

Kalyani, Dayanad, C., Telke, A.A., Govinwar, S.P. and Jadhav, J.P. 2009. Biodegradation and Detoxification of Reactive Textile Dye By Isolated Pseudomonas sp. *Water Environment Research. Proquest Agriculture Journal,*: p298.

Kanu., I. and Achi, O.K. 2011. Industrial Effluent and Their Impect on Water quality of Receiving River in Nigeria. *Journal of Applied Technology in environmental Sanitation,*: 75–86.

Karigar, C., Mahesh, A., Nagenahalli, M. and Yun, D.J. 2006. Phenol degradation by immobilized cells of Arthrobacter citreus. *Biodegradation*. **17**(1): 47–55.

Ketan H., Liang, Z., Guoping, L., Zhaolin, J. and Xu Xiangyang 2009. In-situ bioremediation of polluted source water with simulated river bioreactor. *Journal of Beijing Normal University (Natural Science), China,*. **vol 45,**: pp 295–300, June 2009.

Kevin, B.U. and Xuan, Q. 2001. PCR and sequencing of independent genetic targets for the diagnosis of culture negative bacterial endocarditis. *Diagnostic Microbiology and Infectious Disease.*. **40**((4):): 145–149.

Khan A.G., Kuek, T.., Chaudhry, C. and WJ Hayes. 2000. Role of plants, mycorrhizae and phytochelators in heavy metal contaminated land remediation. *Chemosphere.*. **Vol. 41,**: pp: 197–207.

Kim, K.N., Choi, M.G., Noh, J.H., Ahn, S. and Chang, S. 2008. Rhodamine B Hydrazide Revisited : Chemodosimetric Hg 2 + -selective Signaling Behavior in Aqueous Environments. . **29**(3): 571–574.

Kissi, M., Mountadar, M., Assobhei, O., Gargiulo, E., Palmieri, G. and Giardina, P. 2001. Roles of two white-rot basidiomycete fungi in decolorization and detoxification of olive mill wastewater. *Applied Microbiology and Biotechnology,*. **57**((1- 2)): 221–226.

Kobayashi., F., Maki., T. and Nakamura., Y. 2012. Biodegradation of phenol in seawater using bacteria isolated from the intestinal contents of marine creatures. *International Biodeterioration & Biodegradation*. **69**: 113e118.

Kodori, N.B.M. 2010. *Treatmen of industrial watewater at gebeng area using eichornia crassipes sp. (water hyacinth) pistia stratiotes sp. (water lettuce) and salvinia molesta sp.(giant salvinia).* Universiti Malaysia Pahang.

Korfali, S.I. and Jurdi, M.S. 2011. Speciification of metals in bed sediments and water of Qaraaoun Reservoir, Lebanon. *Journal Environ Monit Assess (2011)*. **178**: 563–579.

Kranz., R., Weston-Hafer., K. and Richards, E. 2006. Identifying Unknown Bacteria Using Biochemical and Molecular Methods. *Howard Hughes Medical Institute (HHMI)*: 2–34.

Kryndushkin, D. 2003. Yeast prion aggregates are formed by small Sup35 polymers fragmented by Hsp104. *Journal of Biological Chemistry.*. **278:** 49–63.

Kuang, Y. 2002. Enhancing Anaerobic Degradation of Lipids in Wastewater by Addition of Co-substrate. . (August).

Kuisiene, N., Raugalas, Er, J., Spro¨, C., Chitavichius., Donaldas, Kroppenstedt and M., R. 2008. Bacillus butanolivorans sp. nov., a species with industrial application for the remediation of n-butanol. *International Journal of Systematic and Evolutionary Microbiology*. **58,**(505–509).

Kumar S, Stohlgren TJ and Chong GW. 2006. Spatial heterogeneity influences native and nonnative plant species richness. *Ecology.*. **87(12):318**: 87(12):3186–99.

Kummerer, K. 2004. Resistence in the environment. *Journal Antimicrob Chemotth*. **45:** 311–320.

Lal, S. and Tabacchioni., S. 2009. Ecology and biotechnological potential of Paenibacillus polymyxa: a minireview. *Indian Journal of Microbiology.*. **49**((1):): 2–10.

Lasheen, M.R. and Ammar, N.S. 2009. Specification of some heavy metals in River Nile sediments, Cairo, Egypt. *Environmentalist,*. **Vol. 29,**: pp: 8–16.

Latha R. and R Kalaivani 2012. Bacterial Degradation of Crude Oil by Gravimetric Analysis. *Pelagia Research Library*. **3**(5): 2789–2795.

Laukova, A., Marekova, M., Vasilkova, Z., Papojava, I. and Juris, P. 2002. Selected microbial consortium of row and digested slurry and its susceptibility to enterocins. *Word Journal of Microbiology and Biotechnology*. **18**: 11–15.

Lee, K., Kim, C.K., Yong, D., Jeong, S.H., Yum, J.H., Seo, Young Hee Docquier, J.-D. and Chong, Y. 2010. Improved performance of the modified Hodge test with MacConkey agar for screening carbapenemase-producing Gram-negative bacilli. *Journal of Microbiological Methods*. **83**: 149–152.

Lee, Y.K. 2006. Microbial Biotechnology principles and applications. USA. *World Scientific Publishing Co.Pte.Ltd. World Scientific Publishing Co.Pte.Ltd.*

Leivouri, M. 1998. Heavy metal contamination in surface sediments in the Gulf of Finland and comparison with the Gulf of Bothnia. *Chemosphere,*. **Vol. 36,**: pp: 723–733.

Leppla, S.H. 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. Proc. Natl. Acad. Sci. USA 79:3162-3166. [PMC free article] [PubMed].

Limited, S.E.S. 2012. *Report review of effective microorganisms (em) and bioaugmentation factors for wastewater and biosolids treatment*.

Lior, H. and Johnson, W.M. 1991. Application of the polymerase chain reaction (PCR) to the aerolysin in whole cellcultures of h-hemolytic Aeromonas hydrophila. *Experientia.*. **47:** 421– 424.

Liu, H. et al. 2004. Formation and Composition of the Bacillus anthracis Endospore. *Journal Bacteriol.*. **186**((1):): 164–178.

Liu Meng 2011. *Identification and partial characterisation of locally isolated lipolytic bacteria*. UNIVERSITI MALAYSIA PAHANG.

Madrid, Y. and Zayas, Z.P. 2007. Water sampling: Traditional methods and new approaches in water sampling strategy. *TrAC Trends in Analytical Chemistry*. **26**(4): 293–299.

Maher, W.A. and Aislabie, J. 1992. Polycyclic aromatic hydrocarbons in near shore marine sediments of Australia. *Science of Total Environment,*. **Vol. 112**((2–3),): pp: 143–164.

Mahmoud, S.A. 2002. *Evaluation of toxycity of some pollutant on histological and biochemical features of orcachomis nilotcus in river nile.* Zagazing University.

Majid, M.A. and Sharma, S.K. 1999. A study of the water quality parameter of the Karnaphuly River. *J. Ban. Chem. Soc.*,. **12**: (1):17–24.

Maliji, D., Olama, Z. and Holail, H. 2013. Original Research Article Environmental studies on the microbial degradation of oil hydrocarbons and its application in Lebanese oil polluted coastal and marine ecosystem. *International Journal of Current Microbiology and Applied Science*. **2**(6): 1–18.

Malik, A. and Ahmed, M. 2002. Seasonal Variation in bacterial flora of the wastewater and soil in the vicinity of industrial area. *Environ monit and assess*. **73:** 263–273.

Margesin, M.R. 2001. Biodegradation and bioremediation of hydrocarbons in extreme environments. : 650–663.

Maryanne B. McGuckin, J.A.P. and Abrutyn, E. 1980. Screening for resistant gram-negative bacteria with gentamicin-MacConkey agar. *American Journal of Infection Control*,. **Volume 8,**(Issue 3,): August 1980, Pages 85–86.

Mat E. A.T., Shaari J. and How V.K. 2011. Wastewater Production, Treatment, and Use in Malaysia. . **2**(4): 256–263.

McMurry, J. and Fay, R.C. 2004. Hydrogen, Oxygen and Water. In: McMurry Fay Chemistry. K.P. Hamann,. : pp: 575–599.

Mendie, U. 2005. The Nature of Water. In: The Theory and Practice of Clean Water Production for Domestic and Industrial Use. Lagos: : pp: 1–21.

Ministry of Health(MOH), M. 2011. Water Supply & Environmental Sanitation (BAKAS) Program Annual Report 2011. *Engineering Services Division, MOH*.

Mohamed, M. 2008. Water Quality Models In River Management. *Proceedings of the 1st Technical Meeting of Muslim Water Researchers Cooperation (MUWAREC)*. **2008**(December): 14–26.

Mohanan, S., Maruthamuthu, S., Muthukumar, N., Rajesekar, A. and Palaniswamy, N. 2007. Biodegradation of palmarosa oil (green oil) by Serratia marcescens. *International Journal of Environmental Science & Technology*,. **4**((2).): pp. 279–283.

Mountouris, A., Voutsas, E. and Tassios, D. 2002. Bioconcentration of heavy metals in aquatic environments: the importance of bioavailability. *Marine Pollution Bulletin*. **44**: pp: 1136–1141.

Muhirwa, D., Nhapi, I., Wali, U.G., Banadda, N., J.J., J.J.K. and Kimwaga, R. 2010. "Characterisation of wastewater from the Nyabugogo Abattoir, Rwanda and the impact on downstream water quality",. *Int. J. Ecol. Develop. Sum.*. **vol. 16,**: no. S10: pp. 30– 46, 2010.

Mukred AM, A., A.-H., A., H. and Yusoff WM. 2008. Growth enhancement of effective microorganisms for bioremediation of crude oil contaminated waters. *Journal Biologi science*. **11(13):** 1708–12.

Munusamy, M. 2010. Urbanisation and water quality control for the source of water in kuala lumpur city. *National Hydraulic Research Institute of Malaysia (NAHRIM)*. (March).

Mvungi, A., Hranova, R.K. and Love., D. 2003. A. Mvungi, R. K. Hranova and D. Love. "Impact of home industries on water quality in a tributary of the Marimba River, Harare: implications for urban water management",. *Phys. Chem. Earth, Parts A/B/C,*. **vol. 28, n**: pp. 1131–1137, 2003.

Najah, A. and Elshafie, A. 2009. Prediction of Johor River Water Quality Parameters Using Artificial Neural Networks. . **28**(3): 422–435.

Nerurkar, A.S. 2007. *Applied microbiology present status of applied microbiology in india*. The Maharaja Sayajirao University of Baroda.

Nhapi, I., Wali, U.G., Uwonkunda, B.K., Nsengimana, H. and Kimwaga., N.B. and R. 2011. Assessment of Water Pollution Levels in the Nyabugogo Catchment, Rwanda. *The Open Environmental Engineering Journal,*. **4;** 40–53.

Nikhil., T., Deepa., V. and Gavankar Rohan., B.S. 2013. Isolation, Characterization and Identification of Diesel Engine Oil Degrading Bacteria from Garage Soil and Comparison of their Bioremediation Potential. *International Research Journal of Environment Sciences.*. **2**((2):): 48–52.

Nriagu, J.O. 1979. Global inventory of natural and anthropogenic emissions of trace metals to the atmosphere. *Nature,*. **Vol. 279,**: pp: 409–411.

Oboh., B.O., Ilori., M.O., Akinyemi., J.O. and Adebusoye., S.A. 2006. Hydrocarbon Degrading Potentials of Bacteria Isolated from a Nigerian Bitumen (Tarsand) Deposit. *Nature and Science.*. **4(3):** 51–57.

Odeyemi, A.T., Aderiye, B.I. and Bamidele, O.S. 2013. Lipolytic Activity of some Strains of Klebsiella , Pseudomonas and Staphylococcus Spp . from Restaurant Wastewater and Receiving Stream. . **3**(1): 43–52.

Okeke BC 2008. Bioremoval of hexavalent chromium from water by a salt tolerant bacterium, Exiguobacterium sp. GS1. *J Ind Microbiol Biotechnol.*. **35**((12):): 1571–1579.

Olayemi, A.B., Igunnugbemi, O.O., Isola, T.M.K. and Eniola, K.I.T. 2008. Response of Resident Bacteria of a Crude Oil-Polluted River to Diesel Oil. *American Eurasian Journal of Agronomy*. **1**(1): 6–9.

Olofsson, I. 2002. *Kartläggning och provtagning av lövskataviken och inre skurholmsfjärden.* Avd. för Tillämpad Geologi. Luleå, Luleå University of Technology.

Omar, A.J. 2010. *Digital modeling evaluation on tidal effect of the galing river. bachelor thesis*. Universiti Malaysia Pahang, Malaysia.

Pan J., Huang., Q. and Zhang., Y. 2004. Gene cloning and expression of an alkaline serine protease with dehairing function from Bacillus pumilus. *Current microbiology*.. **49**((3):): 165–9.

Park, S.-H., Ryu, S. and Kang, D.-H. 2011. Improved selective and differential medium for isolation of Escherichia coli O157:H7. *Journal of clinical microbiology*. **49**(1): 405–8.

Paulse, A., Jacson, V., Khan, S. and Khan, W. 2012. Isolation and identification of bacterial pollutants from the Berg and Plankenburg Rivers in the Western Cape , South Africa. . **38**(5): 819–824.

Pavelic, P., Giordano, M., Keraita, B., Ramesh, V. and Rao, T. 2012. Groundwater Availabelity and use sub Saharan Africa: A review of 15 countries. Colombo, Sri Lanka. *International Water Management Institute (IWMI).*: 274 p.

Perfumo, Amedea, Banat, I.M. and Roger Marchant, and L.V. 2007. "Thermally Enhanced Approaches for Bioremediation of Hydrocarbon-Contaminated Soils." *Chemosphere*. **66:** 179–184.

Pinto, B., Chenoll, E. and Aznar, R. 2005. Identification and typing of food-borne Staphylococcus aureus by PCR-based techniques. *Systematic and Applied Microbiology*. **28**(4): 340–352.

Pohan, A.B. 2010. *Treatment of polluted water (river) by using activated carbon from coir pith.* Technology Bandung Institute.

Pohan, A.B. 2010. *Treatment of polluted water (river) by using activated carbon from coir pith.* Technology Bandung Institute.

Poretz, D.M. 1979. Bacillus anthracis (anthrax), p. 1634-1637. In G. L. Mandell, R. G. Douglas, Jr., and J. E. Bennet (ed.), Principles and practice of infectious diseases.John Wiley and Sons, Inc., New York, NY.

Pritchard, P.H., Mueller, J.G., Rogers, J.C., Kremer, F.V. and Glaser, J.A. 1992. Oil spill bioremediation: experiences, lessons and results from the Exxon Valdez oil spill in Alaska. *Biodegradation*. **3,**: 315–335.

Rahman, K.S.M., Thahira-Rahman, J., Lakshmanaperumalsamy, P. and Banat, I.M. 2002. Towards efficient crude oil degradation by a mixed bacterial consortium. *Bioresource Technology*. **85**(3): 257–261.

Raines, K., Kang, T., Hibbs, S., Cao, G., Weaver, J., Tsai, P., Baillie, L., Cross, A. and Rosen, G. 2006. Importance of Nitric Oxide Synthase in the Control of Infection by Bacillus anthracis. *Infect Immun.*. **74**((4):): 2268–2276.

Rasko, D. 2004. The genome sequence of Bacillus cereus ATCC 10987 reveals metabolic adaptations and a large plasmid related to Bacillus anthracis pXO1. *Nucleic Acids Res.*. **32**((3):): 977–988.

Reid, M.K. and Spencer, K.L. 2009. Use of principal components analysis (PCA) on estuarine sediment datasets: The effect of data pre-treatment. *Journal Environmental Pollution,*. **Vol. 157,**: pp: 2275–2281.

Renzt, R., Widerlund, A., Viklander, M. and Ohlander, B. 2011. Impact of urban stromwater on sediment quality in an Enclosed Bay of the Lule River, Northern Sweeden. *Journal Water Air Soil Pollut.*. **Vol. 218,**: pp: 651–666.

Reynolds, J., Farinha, M. and College, R. 2005. Counting Bacteria. *The Lancet*. **261**(6773): 1239–1240.

Ruyan P, Lianyou, L. and Zhang Hong. 2003. Analysis of The Impacts of Human Activities on Landscape Patterns in Inland River Basin of Arid Zone :Taking the Middle Reaches of HeTian River as an Example,. *Journal of Natural Resources, China,*. **vol. 18 ,**: pp. 492–498,July 2003.

Sabae, S. Z., Erman, W.M. and Rabeh, S.A. 2008. Microbial characteristics of Wadi El-Raiyan Lakes, Egyp. Egypt. *JOurnal Egypt. Academic social Environmental Development. (D-Environmental Studies),*. **9**((2)): 17–28.

Saffran, K., Cash, K., Hallard, K., Wright, B. and R., N. 2001. "CCME Water Quality Index 1.0 User's Manual", Canadian Water Quality Guidelines for the Protection of Aquatic Life, Canadian Environmental Quality Guidelines,. *Canadian Council of Ministers of the Environment,*.

Saher., F.N., Ali., N. binti M., Kadir., T. asmawaty binti abdul, Teruggi., G. and Hossain, mohammed amjed 2012. Environmental Degradation in Malaysia's Pahang River Basin and its Relation with River Pollution: Strategic Plan from Assessment to Mitigation using Geo-Informatics. *International conference on energy, Environment and Sustainable Development, Jamshoro, Pakistan*.

Sandhu, A., Halverson, L.J. and Beattie, G. a 2009. Identification and genetic characterization of phenol-degrading bacteria from leaf microbial communities. *Microbial ecology*. **57**(2): 276–85.

Sandrin, T.R. and Maier, R.M. 2003. Impact of Metals on the Biodegradation of Organic Pollutants. . **111**(8): 1093–1101.

Schwarzbauer, J. and Ricking, M. 2010. Non-target screening analysis of river water as compound related base for monitoring measures. *Research, Environmental Science and Pollution*. **Vol. 17,**: pp: 934–947.

Senthil, R., Angel, K.J., Malathi, R. and Venkatesan, D. 2011. Isolation, identification and computational studies on Pseudomonas aeruginosa sp. strain MPC1 in tannery effluent. *Bioinformation*. **6**(5): 187–90.

Shakeri, A. and Moore, F. 2010. The impact of an industrial complex on freshly deposited sediments, Chener Rahdar river case study, Shiraz, Iran. *Environ Monit Assess.*. **Vol. 169.**: pp: 321–334.

Shalaby, E.A. 2011. Prospects of effective microorganisms technology in wastes treatment in Egypt. *Asian Pacific Journal of Tropical Biomedicine*: 243–248.

Shalaby., E.A. and Nour Sh. El-gendy 2012. Two steps alkaline transesterification of waste cooking oil and quality assessment of produced biodiesel. *International Journal of Chemical and Biochemical Sciences*. **1**: 30–35.

Shokrollahzadeh, S., Azizmohseni, F., Golmohammad, F., Shokouhi, H. and Khademhaghighat, F. 2008. Biodegradation potential and bacterial diversity of a petrochemical wastewater treatment plant in Iran. *Bioresource Technology*. **99**(14): 6127–6133.

Simmons, I.G. 1991. *Earth, air, and water: resources and environment in the late 20th century*. London,: UK: Edward Arnold.

Singh, A., Kumar, V. and JN, S. 2013. Assessment of Bioremediation of Oil and Phenol Contents in Refinery Waste Water via Bacterial Consortium. *J Pet Environ Biotechnoly*. **4:** 145.

Skovbjerg, S. 2010. *Inflammatory mediator response to gram-positive and gram-negative bacteria in vitro and in middle ear infections*. University of Gothenburg.

Snodgrass, D.R. 2013. Successful Economic Development in a Multi-Ethnic Society: The Malaysian Case. Salzburg Seminar. : pp: 1–22.

Sojka, R.E., Upchurch, D.R. and Borlaug, N.E. 2003. Quality Soil Management or Soil Quality Management: Performance Versus Semantic. *Advancey in AgrunoTny*. **79**.

Stabili, L. and Cavallo, R.A. 2011. Microbial pollution indicators and culturable heterotrophic bacteria in a Mediterranean area (Southern Adriatic Sea Italian coasts). *Journal of Sea Research*. **65**(4): 461–469.

Stanley, J.L., Sargeant, K. and Smith., H. 1960. Purification of factors I and II of the anthrax toxin produced in vivo. *J. Gen. Microbiol. [PubMed]*. **22:** 206–218.

Stephen, T.L., Benjamin, Y.P.M., Abudi, M.M. and Joo, H.. 2005. Comparing Activited Sludge and aerobic granulesas microbial inocula for phenol degradation. *Journal Applied Microbiology and Biotechnology*. **67:** 708–713.

Sugai, S.F., Lindstrom, J.E. and Braddock, J.F. 1997. Environmental Influences on the Microbial Degradation of Exxon Valdez Oil on the Shorelines of Prince William Sound, Alaska. *Environ. Sci. Technol.*. **31,**: 1564–1572.

Swannell., Richard P.J, Lee, K., McDonagh and Madeleine 1996. Field Evaluations of Marine Oil Spill Bioremediation. *Microbiological Reviews*. **60,**: 342–365.

Tao L, Hongying, C., Hengzhi, W., Yan, C., Shiming, D. and Wang, L. 2005. Characteristics of the Concentration and Distribution of Heavy Metals in Typical Urban Discharging Pollution River,. *Journal of Basic Science and Engineering, China,*. **No. S1,**: pp. 155–164, October 2005.

Tayel, S.I.. 2002. *Historologycal biochemical and hematological studies on ttilipia zilli and clarios lazera in relation to water quality criteria at different locations in delta berrage.* Branch Zagazing University.

Ting A.S.Y., Tan C. H.C. and Aw, C.S. 2009. Hydrocarbon-degradation by isolate Pseudomonas lundensis UTAR FPE2. *Malaysian Journal of Microbiology*. **5**((2) :): 104–108.

Turner, K., Georgiou, S., Clark, R. and Brouwer, R. 2004. Economic valuation of water resources in agriculture From the sectoral to a functional perspective of natural resource management. *FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS*.

Tusseau-Vuillemin, M. H., Gourlay, C., Lorgeoux, C., Mouchel, J.-M., Buzier, R. and Gilbin, R. 2007. Dissolved and bioavailable contaminants in the Seine river basin. *The Science of the Total Environment,*. **Vol. 375,**: pp: 244–256.

USEPA 2012. *U.s. environmental protection agency, onsite wastewater treatment systems manual*. New York.: USEPA Onsite Wastewater Treatment Systems Manual.

USEPA 1998. *Us environmental protection agency. report of the federal advisory committee on the total maximum daily load (tmdl) program: the national advisory council for environmental policy and technology.* Washington, DC, USA.

Vaalgamaa, S. and Conley, D.J. 2008. Detecting environmental change in estuaries: Nutrient and heavy metal distributions in sediment cores in estuaries from the Gulf of Finland, Baltic Sea. *Estuarine, Coastal and Shelf Science,*. **Vol. 76,**: pp: 45–56.

Valdes, J., Roman, D., Guinez, M., Rivera, L., Morales, T., Avila, J. and Cortes, P. 2010. Distribution and temporal variation of trace metal enrichment in surface sediments of San Jorge Bay Chile. *J. Environ Monit Assess.*. **167**: pp: 185–197.

Vanloon, G.W. and Duffy., S.J. 2005. *The hydrosphere. in: environmental chemistry: a global perspective.* 2nd Edn. New York: Oxford University Press,.

Vazquez, A., M.Costoya, Pena., R.., S.Garcia and C.Herrero. 2003. A rainwater quality monitoring network: a preliminary study of the composition of rainwater in Galicia (NW Spain). *Chemosphere.*. **Vol. 51,**: pp: 375–386.

Venosa, A.D. 2006. NRT Fact Sheet: Bioremediation in Oil Spill Response." EPA, Cincinatti, OH. EPA Office of Emergency and Remedial Response. *Journal of Scientific Research,*. **Ch 8 EPA**.

Walling, D.E. and Fang, D. 2003. Recent trends in the suspended sediments loads of the world's rivers. *Global and Planetary Change,*. **Vol. 39.**: pp: 111 – 126.

Wang, J., Liu, X.D. and Lu, J. 2012. Urban River Pollution Control and Remediation. *Procedia Environmental Sciences*. **13**: 1856 – 1862.

Wang J1., Gao F., Z., L., M., Q., Niu X, KQ, Z. and Huang X. 2012. Pathway and molecular mechanisms for malachite green biodegradation in Exiguobacterium sp. MG2. . **7(12):** 1–27.

Wang, L.K., Chen, J.P., Hung, Y.-T. and Shammas., N.K. 2011. *Membrane and desalination technologie. handbook of environmental engineering:* 13th ed. Humana Press: Springer Science þ Business Media.

Watanapokasin, R.Y., Boonyakamol, A., Sukseree, S., Krajarng, A., Sophonnithiprasert, T., Kanso, S. and Imai., T. 2008. Hydrogen production and anaerobic decolorization of wastewater containing Reactive Blue 4 by a bacterial consortium of Salmonella subterranea and Paenibacillus polymyxa. *Journal Biodegradation.*. **20**((3):): 411–418.

Wheeler, D., Chappey, C., Lash, A., Leipe, D., Madden, T., Schuler, G., Tatusova, T. and Rapp, B. 2000. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res.*. **28**((1):): 10–4 [PubMed].

WHO and (World Health Organization). 2004. Guidelines for drinking- water quality, third edition, incorporating first addendum (third edition).

Widjojoatmodjo, M.N., Fluit, A.D.C. and Verhoef, J.A.N. 1994. Rapid identification of bacteria by Rapid Identification of Bacteria by PCR-Single-Strand Conformation Polymorphism. . **32**(12).

Wu, L., Luo, Y.P., Wan, J.B. and Li, S.G. 2006. Use of Yarrowia lipolytica for the treatment of oil/grease wastewater. *Research of Environmental Science (China),*. **19**((5)): 122–125.

Xiang, H., Lu, X., Yin, L., Yang, F., Zhu, G. and Liu, W. 2013. Microbial community characterization, activity analysis and purifying efficiency in a biofilter process. *Journal of Environmental Sciences*. **25**(4): 677–687.

Xiao, Z. 2009. Acetoin Catabolism and Acetylbutanediol Formation by Bacillus pumilus in a Chemically Defined Medium. *journal PLoS ONE.*. **4**((5):): 5627–5635.

Yakrus, M.A., Hernandez, S.M., Floyd, M.M., Sikes, D., Butler, W.R. and Metchock, B. 2001. Comparison of methods for identification of Mycobacterium abscessus and M. chelonae isolates. *J Clin Microbiol.*. **39**: 4103–4110.

Yongming Z., Yizhen, H., Rong, Y. and Liu Fang. 2009. Anoxic Bioremediation of Urban Polluted River Water with Biofilm,. *Environmental Science, China,*. **vol 30 ,**: pp 1920–1924 ,JUL 2009.

Zaid, A., Hughes, H.G., Porceddu, E. and Nicholas, F. 2001. Glossary of Biotechnology for Food and Agriculture - A Revised and Augmented Edition of the Glossary of Biotechnology and Genetic Engineering. *Food and Agriculture Organization of the United Nations*. **9**.

Zhang., G., Yue-ting., W., And, Q.X. and Qin, M. 2005. Biodegradation of crude oil by Pseudomonas aeruginosa in the presence of rhamnolipids. *Journal of Zhejiang University SCIENCE*. **6B**((8)): 725–730.

Zhou, H.Y., Peng X.T. and Pan, J.M. 2004. Distribution, source and enrichment of some chemical elements in sediments of the Pearl River Estuary,. *China. Cont. Shelf Res,*. **Vol. 24,**: pp: 1857 – 1875.

Zhu, X., Venosa, A., Suidan, M. and Lee., K. 2001. Guidelines for the Bioremediation of Marine Shorelines and Freshwater Wetlands.U.S. EPA, Cincinatti, Ohio. *Journal of Environmental Pollution,*: 6–7.

APPENDICES

APPENDIX A

INTERIM NATIONAL WATER QUALITY STANDARDS FOR MALAYSIA (INWQS)

Parameters	(Units)	Classes				
		I	IIA / IIB	III[@]	IV	V
A1	mg/l		-	(0.06)	0.5	
As	mg/l		0.05	0.4 (0.05)	0.1	
Ba	mg/l		1	-	-	
Cd	mg/l		0.01	0.01* (0.001)	0.01	
Cr(VI)	mg/l		0.01	1.4 (0.05)	0.1	
Cr(III)	mg/l	N	0.05	2.5	-	L
Cu	mg/l		1	-	0.2	
Hardness	mg/l	A	250	-	-	E
Ca	mg/l		-	-	-	
Mg	mg/l		-	-	-	V
Na	mg/l	T	-	-	3 SAR	E
K	mg/l		-	-	-	
Fe	mg/l	U	0.3	1	1 (leaf) 5 (others)	L
Pb	mg/l	R	0.05	0.02* (0.01)	5	S
Mn	mg/l		0.1	0.2	0.2	
Hg	mg/l	A	0.001	0.004 (0.0001)	0.002	
Ni	mg/l		0.05	0.9*	0.2	A
Se	mg/l	L	0.01	0.25	0.02	
Ag	mg/l		0.05	0.0002	-	B
Sn	mg/l		-	0.004	-	
U	mg/l		-	-	-	O
Zn	mg/l		5	0.4*	2	
B	mg/l		1	(3.4)	0.8	V
Cl	mg/l		200	-	80	
Cl_2	mg/l	L	-	(0.02)	-	E
CN	mg/l		0.02	0.06 (0.02)	-	
F	mg/l	E	1.5	10	1	
NO_2	mg/l		0.4	0.4 (0.03)	-	
NO_3	mg/l		7	-	5	IV
P	mg/l	V	0.2	0.1	-	
Si	mg/l		50	-	-	
SO_4	mg/l	E	250	-	-	

S	mg/l	L	0.05	(0.001)	-
CO_2	mg/l		-	-	-
Gross-a	Bq/l		0.1	-	-
Gross-b	Bq/l		1	-	-
Ra-266	Bq/l		< 0.1	-	-
Sr-90	Bq/l		< 1	-	-

* = At hardness 50 mg/l $CaCO_3$
\# = Maximum (unbracket) and 24 - hour average (bracketed) concentrations
N = Free from visible film sheen, discoloration and deposits

Note:-

@ Maximum (unbracketed) and 24-hour average (bracketed) concentrations

Class	Uses
l	Conservation of natural environment
	Water supply l - practically no treatment necessary (except by disinfection of boiling only) Fishery l - very sensitive aquatic species
llA	Water supply ll - conventional treatment required
	Fishery ll sensitive aquatic species
llB	Recreational use with body contact
lll	Water supply lll - extensive treatment required
	Fishery lll - common, of economic value and tolerant species
lV	Irrigation
V	None of the above

APPENDIX B

LIST OF PUBLICATIONS

1. POSTER PRESENTATION (2012)

   Mupit Datusahlan, W.M.F. Wan Ishak, Essam A Makky. Biological, Physical and Chemical Characterization Water Quality Parameter of Galing River. Presented at the 5th International Conference on Postgraduate Education (ICPE-5th 2012), 18-19 December, 2012, Dewan Sultan Iskandar (DSI), Universiti Teknologi Malaysia (UTM), Johor Bahru, Malaysia

   Mupit Datusahlan & Wan Mohd. Faizal Bin Wan Ishak. *Case Study: Water Quality Parameters To Determine Quality of Water Galing River Kuantan With Defferent Location Plot*. Presented at National Conference on Postgraduate Research 2012 (NCON-PGR2012), 8-9 September, 2012, Universiti Malaysia Pahang (UMP), Gambang, Pahang, Malaysia.

2. ORAL PRESENTATION (2013)

   Mupit Datusahlan, W.M.F. Wan Ishak, Essam A Makky. Identification and Characterization of Bacterial Potential to Degrade Wastewater Oil Pollutants: A Case Study - Galing River, Kuantan Pahang, Malaysia. Presented at the 1st Indonesian Student Conference on Science and Mathematics (ISCSM-1) 2013, 24-25 July, 2013, Gedung Kuliah Umum (GKU Timur), Building, Institute Technology Bandung (ITB), Indonesia.

3. JOURNAL PUBLICATION

   Mupit Datusahlan, W. M. F. Wan Ishak, and Essam A. Makky (2013): "Biodegradation of Wastewater Oil Pollutants, Identification and Characterization: A Case Study–Galing River, Kuantan Pahang, Malaysia," *International Journal of Bioscience, Biochemistry and Bioinformatics* (IJBBB) Vol. 3(6): 579-582 ISSN: 2010-3638 DOI: 10.7763/IJBBB.2013.V3.280,http://www.ijbbb.org/show-42-569-1.html

APPENDIX C

COMMERCIAL KITS FOR PCR

G.1: G-Spin Genomic DNA Extraction Kit (For Bacterial) (iNtRON Biotechnology, INC, 2010)

Kit Contents: Pre-buffer, G-buffer, Binding Buffer, Washing Buffer A, Washing Buffer B, Elution Buffer, Columns Containing Silica-Membrane, Collection Tubes (Polypropylene Tube), Lysozyme Stock Solution, Proteinase K Stock Solution, Proteinase K Stock Solution.

G.2: MEGA quick-spin PCR & Agarose gel Extraction System (iNtRON Biocenology, INC, 2010)

APPENDIX D

1 KB PLUS DNA LADDER STANDARD PICTURE

D.1:

1 Kb Plus DNA Ladder
0.7 µg/lane
0.9% agarose gel
stained with ethidium bromide

Structure of Fragments in 1-Kb Increments:

5′-GATCC——— G
G ——— CCTAG-5′
(996 bp)$_n$

Notes:
During 1% agarose gel electrophoresis with Tris-acetate (pH 7.5) as the running buffer, bromophenol blue migrates together with the 500 bp band.

The 1650 bp band is generated from pUC. The bands smaller than 1000 bp are derived from lambda DNA.

APPENDIX E

GRAM -STAINING REAGENTS

B.1: Crystal violet

Crystal violet 85% dye	2 g
95% ethyl alcohol	20 ml

Mix and dissolve.

Ammonium oxalate	0.8 g
Distilled water	80.0 ml

B.2: Gram's iodine solution

Iodine crystals	1 g
Potassium iodide	2 g
Distilled water	300 ml

Store in an amber bottle; discard when the color begins to fade.

B.3: Safranin solution

Safranin	2.5 g
95% ethyl alcohol	100.0 ml

For a working solution, dilute stock solution 1/10 (10 ml of stock safranin to 90 ml of distilled water).

APPENDIX F

KIT COMPONENT GF-1 BACTERIAL DNA EXTRACTION KIT

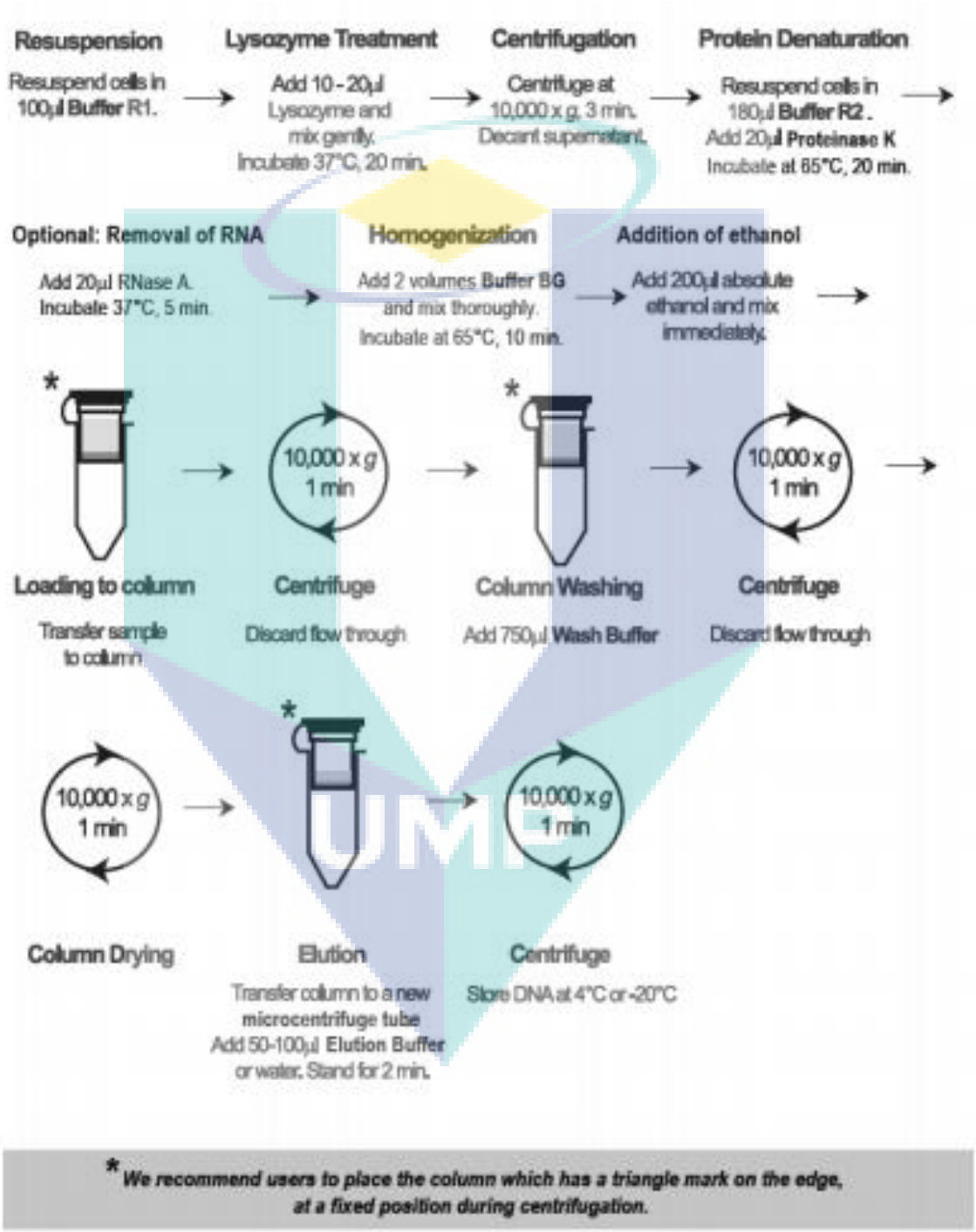
Kit component

Product Catalog No.	5 Preps SAMPLE	50 Preps GF-BA-050	100 Preps GF-BA-100
Components			
GF-1 columns	5	50	100
Collection tubes	5	50	100
Resuspension Buffer 1 (Buffer R1)	1 ml	8 ml	15 ml
Resuspension Buffer 2 (Buffer R2)	1.5 ml	12 ml	24 ml
Bacterial Genomic Binding Buffer (Buffer BG)	4 ml	28 ml	56 ml
Wash Buffer	2.4 ml	17 ml	34 ml
Elution Buffer	1.5 ml	10 ml	20 ml
Proteinase K*	0.11 ml	1.05 ml	2 x 1.05 ml
Handbook	1	1	1

* Please refer to Reconstitution of Solutions and Storage and Stability before using this kit.

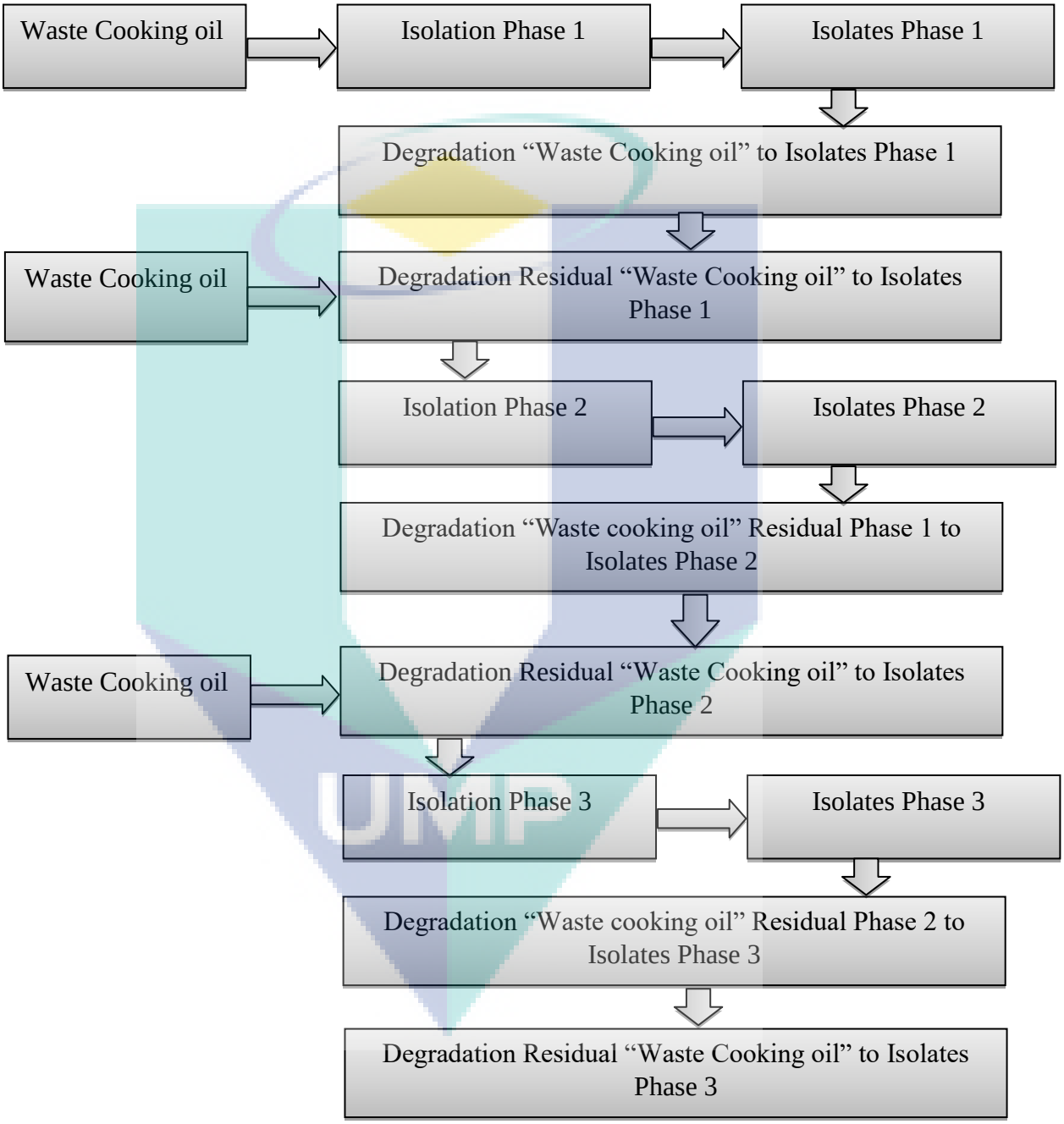
APPENDIX G

FLOW CHAT METHOD BACTERIAL DNA EXTRACTION



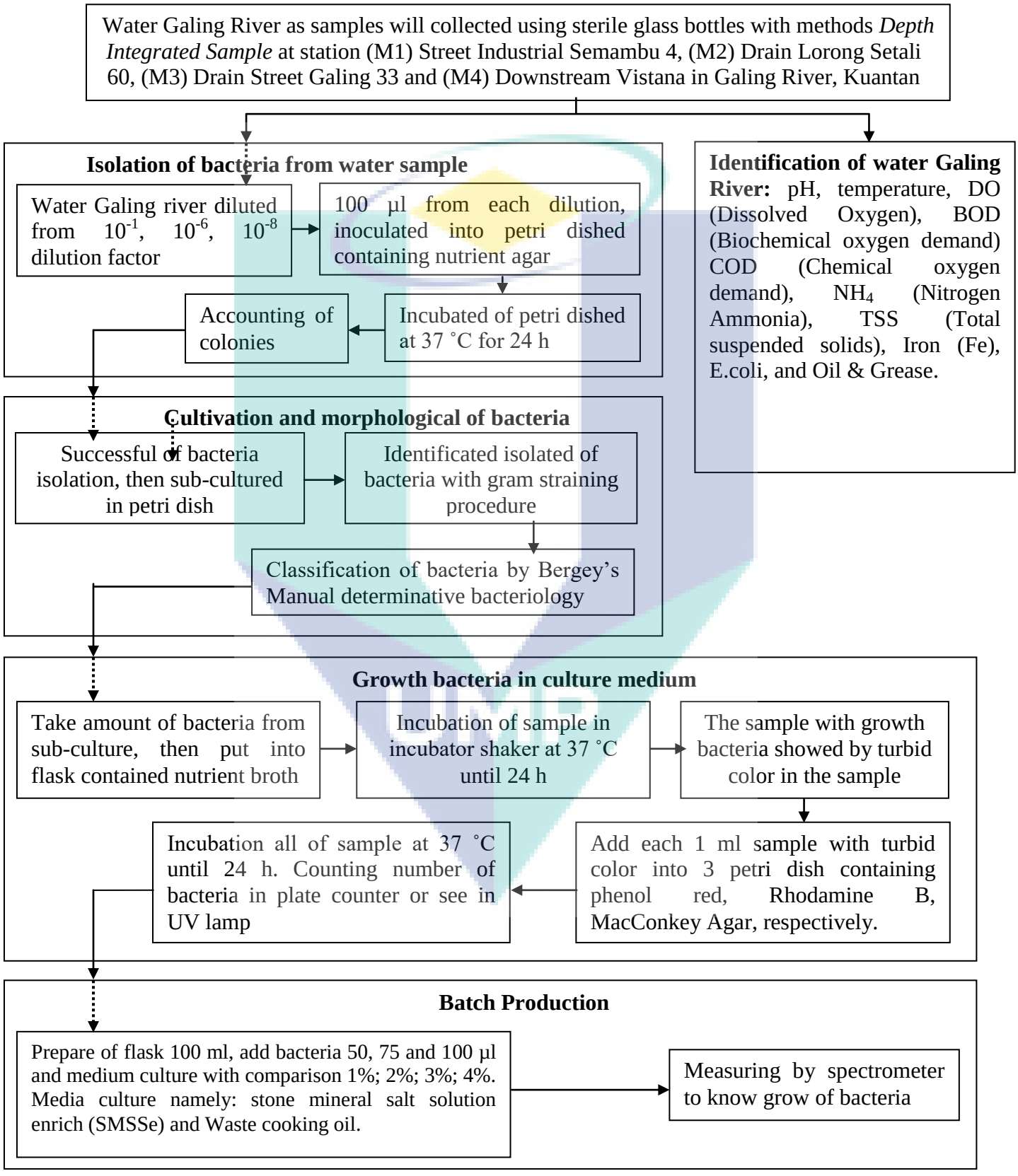
APPENDIX H

FLOW CHAT SCHEMATIC PROCESSES STEP APPLICATION BATCH PRODUCTION

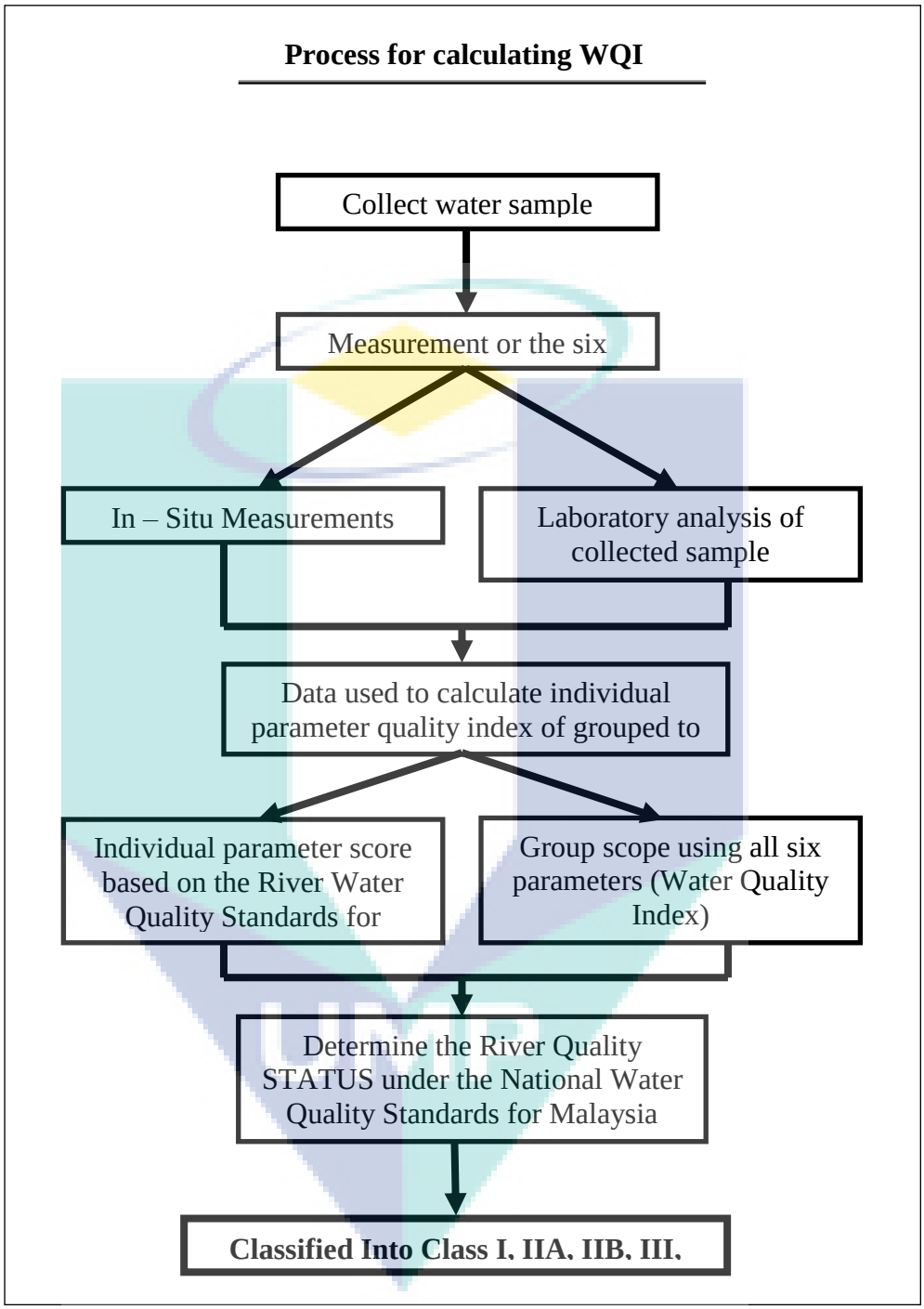


APPENDIX I

FLOW CHART METHOD THE OVERALL PROCEDURE DEPICTED IN PRESENT STUDY A FLOW CHART IS GIVEN BELOW



ATTACHMENT


Process for calculating WQI
Collect water sample
Measurement or the six
In – Situ Measurements
Laboratory analysis of collected sample
Data used to calculate individual parameter quality index of grouped to
Individual parameter score based on the River Water Quality Standards for
Group scope using all six parameters (Water Quality Index)
Determine the River Quality STATUS under the National Water Quality Standards for Malaysia
Classified Into Class I, IIA, IIB, III,