EFFECT OF ENDOGENEOUS SOIL HUMIC ACIDS ON MICROBIAL FE(III) REDUCTION

NG ZHI LEI

F15A0102

A thesis submitted in fulfilment of the requirements for the degree of Bachelor of Applied Science (Bioindustrial Technology) with Honours

Faculty of Bioengineering and Technology

Universiti Malaysia Kelantan

2019

DECLARATION

I hereby declare that the work embodied in this report of the original research and has not been submitted for a higher degree to any universities or institutions.

Signature

Student Name:

Matrix No:

Date:

Approved by:

Supervisor's Signature

Name:

Chop

Date:

AKNOWLEDGEMENT

First of all I would like first thank my Supervisor FYP, Dr. Wee Seng Kew for his encouragement, guidelines and critics toward this project from the beginning until the end of this project. Besides, he always motivated me through his kind words, which urge me to do my best in order to achieve my target in this project and also had given me a lot of advices and information on this project to ensure all on the right track. There is no regret for me to say that he had played his role well.

Appreciation to all my dear members, Tan Hui Ying, Lee Chun Eng and Teng Pei Pei for all the tutoring and spending precious time with me during this period. Without their help and tolerance, I would not be able to complete this project successfully.

In addition, I would also express my deepest thanks to my beloved family for their caring, encouragement and support both financially and moral that made me manages to finish this project.

Last but not least, I would like to thanks to all crews that involved either directly and indirectly to this project. A special thanks to University Malaysia Kelantan for providing me many information and guidance to complete this project.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

^{o}C	Degree celcius
mM	milliMolar
mL	Millilitre
nm	Wavelength
µl	Microliter
mg	Milligram
M	Molar

LIST OF ABBREVIATIONS

$CaCl_2•2H_2O$	Calcium Chloride Dihydrate
$COSO_4.7H_2O$	Cobalt (II) Sulphate Heptahydrate
$CuSO_4•5H_2O$	Copper (II) Sulphate
EDTA	Disodium Salt
Fe (III)	Ferric Iron
Fe (II)	Ferrous Iron
Ferrozine	Monosodium salt hydrate of 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid
$FeSO_4•7H_2O$	Iron (II) Sulphate Heptahydrate
H_3BO_3	Boric Acid
HFO	Hydrous Fe (III) Oxide
K_2HPO_4	Dipotassium Phosphate
KH_2PO_4	Monopotassium Phoshate
LB	Luria-Bertani Medium
$MgSO_4•7H2O$	Magnesium Sulphate Heptahydrate
$MnSO_4•H_2O$	Manganese (II) Sulphate Monohydrate
$Na_2MoO_4•2H_2O$	Sodium Molybdate Dihydrate
$NaHCO_3$	Sodium Bicarbonate
NaCl	Sodium Chloride
$NaSeO_4$	Sodium Selenite Solution
$(NH_4)_2SO_4$	Ammonium Sulphate
$NiCl_2.6H_2O$	Nickel (II) Chloride Hexahydrate
$ZnSO_4•7H_2O$	Zinc Sulphate Heptahydrate

The Effect of Endogeneous Soil Humic Acids on Microbial Fe(III) Reduction

ABSTRACT

Humic substances are fraction of soil organic matter which widespread in nature. Humic acids (HAs) are one of the most constituent of humic substances except from fulvic acids and humin. Dissimilatory metal-reducing bacterium, *Shewanella oneidensis* strain MR-1 utilizes humic acids as electron shuttle for Fe(III) reduction. The presence of HAs humic acids enhances the microbial Fe(III) reduction activities. Reduction of insoluble Fe(III) oxides into soluble Fe(II) increase bioavailability of Fe in soil and could enhance absorption of Fe by plants. Aim of this study is to investigate the effect of endogeneous soil HAs on microbial Fe(III) reduction. HAs were extracted from soil samples collected from 12 locations which comprise of palm oil plantation, paddy field, pineapple plantation, agriculture land and rainforests. The effects of the extracted HAs on Fe(III) reduction activity by *S.* oneidensis MR-1 were determined by adding the extracted HAs into M1 minimum media supplemented with lactate as the sole electron donor and Fe(III) hydroxyoxides (HFO) as the sole electron acceptor. In the presence of humic acids, microbial Fe(III) reduction significantly increase by functioning as electron shuttle between microorganisms and ferrihydrite anaerobically. FTIR spectrum describe that HAs extracted from 12 locations have similar functional groups but with different strength of bonding. In summary, this study reveal that humic acids is greatly impact on microbial Fe(III) reduction. The mechanism of humic acids as electron shuttle need to be applies in agriculture uses.

Keywords: Humic acids, *Shewanella oneidensis* strain MR-1, Fe(III) reduction, electron shuttling, concentration of Fe(II)

Pengaruh Asid Humik terhadap Mikrobial Fe(III) Pengurangan

ABSTRAK

Bahan-bahan humik adalah pecahan bahan organik tanah yang dikandungi dalam alam semula jadi. Asid humik adalah salah satu daripada unsur-unsur humik yang paling banyak kecuali daripada asid fulvic dan humin. *Shewanella oneidensis* strain MR-1 sebagai bakteria pengurangan logam yang disimilatory membolehkan asid humik berfungsi sebagai pengangkutan elektron dalam pengurangan Fe(III). Kehadiran asid humik HA meningkatkan aktiviti pengurangan Fe(III) mikrobial. Pengurangan oksida Fe(III) kepada Fe(II) yang lebih larut membantu meningkatkan bioavailabiliti Fe di dalam tanah dan dapat meningkatkan penyerapan Fe oleh tumbuhan.Tujuan kajian ini adalah untuk mengkaji kesan asid humik (HA) terhadap pengurangan mikrobial Fe(III). Untuk menentukan keberkesanan asid humik terhadap pengurangan mikrobial Fe(III), 12 sampel tanah dari ladang sawit, medan padi, tanah pertanian dan hutan telah dikumpulkan. Kesan HA yang diekstrak terhadap aktiviti pengurangan Fe(III) oleh *S. oneidensis* MR-1 telah disiasat dengan penambahan HA yang diekstrak ke dalam media minimum M1 dengan laktat sebagai penderma elektron tunggal dan Fe(III) hydroxyoxides (HFO) sebagai penerima elektron tunggal. Dengan kehadiran HA, pengurangan Fe(III) mikrobial meningkat secara signifikan kerana HA berfungsi sebagai pengangkutan elektron antara mikroorganisma dan ferrihidrit secara anaerobik. Spektrum FTIR menerangkan bahawa HA yang diekstrak daripada 12 lokasi mempunyai kumpulan fungsi yang sama tetapi mempunyai kekuatan ikatan yang berlainan. Secara ringkasnya, kajian ini mendedahkan bahawa HA dapat mempengaruhi pengurangan Fe(III) mikrobial. Mekanisme HA sebagai pengangkutan elektron boleh digunakan dalam sektor pertanian.

Kata kunci: Asid humik, strain *Shewanella oneidensis* MR-1, pengurangan Fe(III), pengangkatan elektron, kepekatan Fe(II)

CHAPTER 1

INTRODUCTION

1.1 Research Background

Humic acids are macromolecules and insoluble in low pH <2 but soluble in alkaline condition (McCarthy & Bronk, 2008). Forms of humic acids are due to chemical and biological humification of natural matter present in the soil with helps of biological activities of microorganisms. Their features are based on types of organic matters undergo decomposition, forming duration and environment factors.

Elemental analysis of humic acids consists of carbon, oxygen, hydrogen, nitrogen, phosphorus and sulphur (Brondi et al., 2016). Body of humic acids consist of aromatic rings which derived from phenols and quinones synthesized by microorganisms (Fenchel, King, & Blackburn, 2012). Existence of phenolate and

carboxylate groups enables humic acids forming of chelate complexes with ions which aid in regulating bioavailability of metal ions (Pettit, 2012).

Redox reactive and recyclable properties serve as the criteria of humic acids become an electron shuttle. Electron shuttle plays a vital role in microbial Fe(III) reduction. During reduction, humic acids serve an electron donor which receive electron from microorganism. Humic acids being reduced, after receive electron from microorganism. The reduced humic acids then transfer this electron to Fe(III). After that, Fe(III) is reduced to Fe(II) by microbial reduction process (Derek, Elizabeth L, Blunt-Hrris, Elizabeth J.P & John C, 1996).

1.2 Problem Statement

Iron is essential nutrient for plant growth. In nature, availability of iron for plant absorption is limited. Fe(II) has high solubility for plant nutrition absorption whereas Fe(III) has lower. Solubility of organic materials in soil is important which gives plants growth healthier and lush. However, Fe(III) is generally found in nature than Fe(II).

Iron is essential for plant growth such like involved in chlorophyll synthesis (Gyana R. Rout et al.,2015). However, iron deficiency is a common nutritional deficiency phenomena arise in agriculture sector which resulting in poor yields and reduced nutritional quality. Chlorosis is the plant disease which deficiency of iron compound in plant cells. The major symptom is yellowing of the plant leaves.

Conventional product such as Forliar spray was developed to treat this chlorosis. However, this technology is not efficient and only temporally cures this disease due to its only spraying the Forliar on the surface of leaves.

To consistently treat this problem, conversion mechanism of unavailability iron to availability iron in soil should be study and apply for agriculture growth purpose. Humic acids constitute the most abundant organic fraction in the biosphere which consist redox active functional groups such as quinones and enable form the humic metal complexes. Thus, iron-reducing bacteria such as *S. oneidensis* MR-1 could use humic acids as electron acceptor for respiration. Then, the reduced humic acids would transfer the electron to ferric iron as Fe(III) reduction.

1.3 Objectives

- To extract soil humic acids from various soil samples.
- To investigate the effect of endogeneous soil humic acids on microbial Fe(III) reduction.
- To characterize humic acids from various source of soil samples.

1.4 Scope of Study

Humic acids are extracted from a wide diversity of organic rich environments, including soil from rainforest, palm oil plantation, pineapple plantation, vegetable garden and paddy field. Extracted humic acids will characterized by using spectrophotometer (Shirshova, Gilichinsky, Ostroumova, & Yermolayev, 2015) and fourier-transform infrared spectroscopy (FTIR). Effect of endogeneous soil humic acids on microbial Fe(III) reduction will be determined through Ferrozine assay test.

1.5 Significance of Study

Types of nutrients present in the soil are huge and important for plants to growth, metabolism or external supply. However, nutrients could be absorbed by plant as nutrient sources are limited due to the bioavailability. Fe(III) is one of the nutrients needed by plants to growth but it has low solubility for plant nutrient absorption. Thus, mechanism of conversion from unavailability of Fe(III) to bioavailability source for plant absorption is important with the help of humic acids. The unique composition of humic acids allows it serve as electron shuttle on microbial Fe(III) reduction.

By understanding the mechanisms, soil fertility can be improved by increase bioavailability of nutrient in soil. The increase of soil fertility helps to increase the plant nutrient absorption. Thus, indirectly increase the yield of agriculture product as well. Besides of increasing the yield, humic acids also help to mediator the soil condition

which making plants look lush greenery and growing luxuriantly. As a nutshell, increase the yield of agriculture product helps to solve food shortage around the world with the greatly increasing of the population.

CHAPTER 2

LITERATURE REVIEW

2.1 Soil Organic Matter

Soil organic matter is the fraction of the soil which derived from decomposition of plant or animal tissue. Most of productive agricultural soils have between 3 and 6% organic matter (Megan Fenton, 2008). In neutral and alkaline soils, organic matter such as humic acids (HAs) and humin are majority present in the soil (Pettit, 2012).

2.1.1 Humic Acids

Organic matter in soil is a key factor and known to regulate and improve the soil properties in chemical, biological and physical functions. Humic acids has the average chemical formula $C_{187}H_{186}O_{89}N_9S_1$ and is insoluble in strong acid (pH = 1). Humic acids are natural acidic organic polymer that can be extracted from humus found in nature. It considers final breakdown constituents through the chemical and biological humification of plant and animal matter or biological activities of microorganism. The high mineralization of humic acids contributes to soil fertility. In soil, humic acids chelate nutrient compounds such like iron to provide plant absorption and nutrition. Thus, optimized nutrient supply and increase the yield of plants.

Humic acids are the organic materials that coagulate when the strong-base extract is acidified while fulvic acids are the organic materials that remain soluble when the strong-base extract is acidified (Bleam, 2017). Usually, humic acids consist of aromatic compounds and aliphatic compounds in the structure. In organic chemistry, both of these compounds are under hydrocarbon. Nonetheless, both have different properties such as aromatic compound consist of a stable ring whereas aliphatic compound consider open ring compound which does not have ring in the structure.

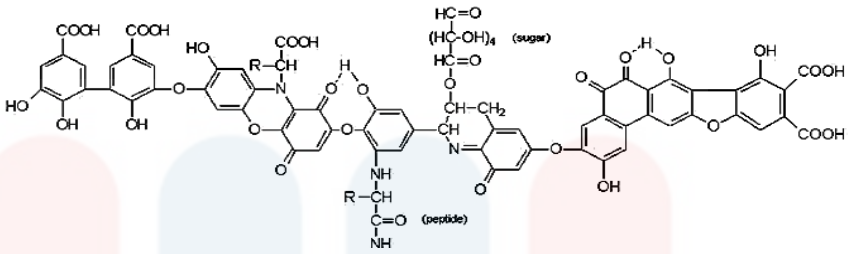


Figure 2.1: Model structure of humic acids (Source:(K. Kumada, 1987)

Properties of humic acids are depend on vary characteristics such as climate, soil source and others. Hence, different origins of soil give different properties of humic acids. Study from Tsutsuki & Kuwatsuka (1979) showed that quinone and functional groups of humic acids such as carboxyls, phenolic, alcoholic hydroxyls and carbonyls group are contribute to light absorption of humic acids in the visible region. However, different type of humic acids will content varies type of functional group and result in diverge range of participation.

In the carbon biogeochemical cycle, the transformation of living organic substances into humic substances or humification is vital (Purmalis & Klavins, 2013). It is well known that humification of humic acids is an early stage of carbonization at ordinary temperature and pressure with presence of oxygen and moisture (K. i. Kumada, 1987). Carbonization is a process of conversion for organic substances into carbon or carbon containing residue. During decomposition of plant and animal residues, bulk of the material is converted to carbon dioxide, water and energy through enzymatic oxidation. Subsequently, elements will be released or immobilized to the earth likes nitrogen, phosphorous and sulfur (Friedhelm G, 2006). Thus, humic acids consider as a

product of low temperature natural carbonization of organic materials (Baskakov et al., 2016).

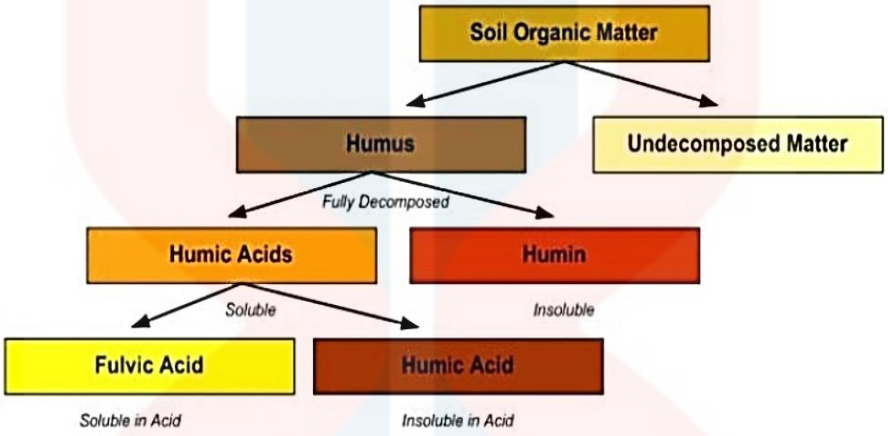


Figure 2.2: Humus synthesis (Source: (Siddharth Natarajan, 2016)

The diverse range of conjugated double bond presence in the humic acids structure give its brown in color and this chemical configurations also influence the light absorption of humic acids in spectrophotometer. The formation of this conjugated double bond system is due to nature occur humification. Humification is a process that occurs in soils and peats which organic material decompose and break down. There classified 2 layer of soil during sampling which are topsoil and deep soil. Topsoil also known as acrotelm layer which the place aeration triggers active decomposition of the peat while cellulose and lignin of plant tissue break down into humic acids. On the other hand, deep soil known as catotelm layer. In this layer, the soil lies below the water table which makes it continually saturated. After that, the peat will become very dense (Cassandra Worman et al., 2002). Thus, deep soil should be collected during sampling due to its high humification level. Higher the level of peat break down, the more humic

acids will be produced. The humification level of soil can be achieved by measure it color in spectrophotometer (J. A. Srnnms et al., 1968).

Figure 2.3: Acrotelm and catotelm (Source: Cassandra Worman et al., 2002)

2.1.2 Fulvic Acids

Fulvic acids are found in nature as a final product of microbial metabolism processes. It also contributes huge in soil fertilization and agriculture supplementation by increasing permeability of plant membrane during plant nutrition. Sizes of the fulvic acids are much smaller than humic acids but it also consider as a biologically active molecule. It serves as same function with humic acids which intermediate chelators to form chelates complex with metal ions. However, it has low phenols and aromatic compared to humic acids.

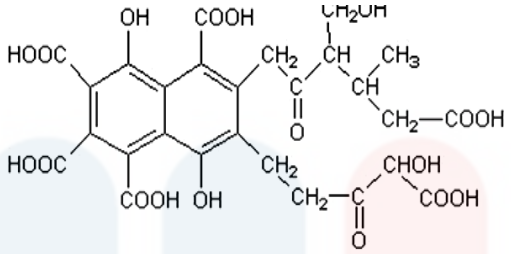


Figure 2.4: Model structure of fulvic acids (Source: (Wang et al., 2006)

2.1.3 Humins

Humins are the fraction humic substances which did not soluble in water at any pH. It has slow breakdown rate or high resistant to decomposition compared to humic acids and fulvic acids. However, it enable maintain soil stability and improve soil's water holding capacity as long as improve soil fertility through cation exchange system (Pettit, 2012).

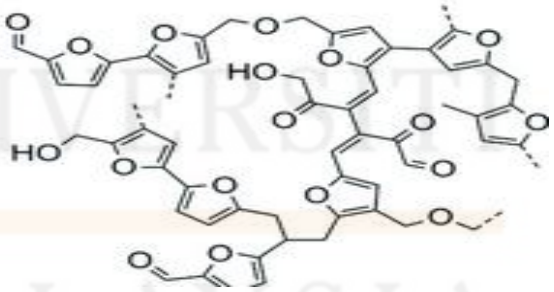


Figure 2.5: Model structure of humins (Source: (Wang et al., 2006)

2.2 Dissimilatory Fe(III) Reducing Bacteria (FERB)

Iron reducing bacteria (FeRB) play an important role in iron reduction in soil which using ferric iron as electron acceptor under anaerobic conditions. By using FeRB, iron reduction can be develop through respiration (as the electron acceptor) or fermentation (as an electron sink). The involvement of FeRB in redox processes crucial to regulating environmental biogeochemical processes (Bocanegra, Lobartini, & Orioli, 2006).

2.2.1 *Shewanella oneidensis* MR-1

Shewanella oneidensis MR-1 is Gram-negative, facultative anaerobes and belongs to the class Gammaproteobacteria. It mostly distributed in soil environment, marine, sedimentary and freshwater. This microorganism is adopted in studying bacterial metabolic and regulatory systems that facilitate their survival in redox-stratified environments. Many research noted that *S. oneidensis* MR-1 has ability to utilize variety of electron acceptors for respiration which include soluble organic and inorganic compounds. The present of cytochrome in *S. oneidensis* MR-1 structure which has ability to reduce heavy metals in the environment has been target in bioremediation study (Kasai, Kouzuma, & Watanabe, 2017).

2.3 Extracellular Electron Transfer

Fe(III) in soil can be mobilized from anaerobic environments through the activity of extracellular electron transfer by dissimilatory metal-reducing bacteria. The dissimilatory metal-reducing bacteria are use of Fe(III) as a terminal acceptor to generate energy during anaerobic respiration. There have several mechanism was developed by the dissimilatory metal-reducing bacteria in order to transfer electron extracellularly including direct contact, nanowires, and electron shuttling (Gralnick, 2013).

2.3.1 Direct Contact Reduction

Dissimilatory metal-reducing bacteria able to utilize a direct contact mechanism for extracellular electron transfer. In this case, electron shuttles for reduction of metal oxides are not required. The metal reducing pathway of *S.oneidensis* MR-1 involve of 6 multihaem c- Cyts which are Cym A, Fcc3, MtrA, MtrC, MteB, OmcA and small tetrahaem cytochrome (STC). Each of the multihaem has its own functional properties and this Mtr pathway consider a finest microbial extracellular electron transfer pathway in existing knowledge. To outline, cytochromes are molecule found in *S.oneidensis* MR-1 and function as a Fe (III) reductase. Meanwhile, Cym A, Fcc3, MtrA, MtrC, MteB, OmcA and (STC) form a reducing pathway that oxidizes quinol in cytoplasmic membrane. Then, the released electron will across the entire cell envelope to the surface of minerals (Shi et al., 2016).

2.3.2 Nanowires Electron Transportation

Bacterial nanowires offer an extracellular electron transport pathway for linking the respiratory chain of bacteria to external surfaces such as oxidized Fe(III) in the environment. It is formed by protein filaments and anchored in the cell envelope (Shi et al., 2016). *S.oneidensis* MR-1 nanowires are membrane based which is much different with the *Geobacter* nanowires are thought to be type IV pili. *S.oneidensis* MR-1 nanowires contain multiheme cytochromes and are associated with outer membrane vesicles (Pirbadian et al., 2014). With nanowires, electrons can be transfer electrons to an electron acceptor that is not in direct contact with the cell but rather located distantly from it (Bucking, Piepenbrock, Kappler, & Gescher, 2012).

2.3.3 Electron Shuttling Pathway

Electron shuttle has capability transfer electron from electron donor to electron acceptor which translocate from one location to another. Humic acids were shown to be redox-active and able contribute in relevant abiotic redox-reactions due to its aromatic and quinone structures (Xu, 2009). The redox reactive and electron shuttling function of humic acids were studied with *Shewanella oneidensis* MR-1 as driving force of electron transfer. During microbial metal respiration, *S. oneidensis* MR-1 is expected transfer electron to humic acids. Then, this reduced humic acids are capability stimulate Fe(III) reduction. Thus, humic acids serve as electron shuttle when receive electron from *S.*

oneidensis MR-1 and donate it to Fe(III). Electron shuttle helps in accelerate Fe(III) reduction due to allay the Fe(III) reducer directly contact and reduce the Fe(III) oxides (Derek, Elizabeth L, Blunt-Hrris, Elizabeth J.P & John C, 1996).

2.4 Application of Humic Acids

Presence of quinone, carboxylate and phenolate groups gives the humic acids the ability to form (chelate) complexes with ions such as Mg^{2+}, Ca^{2+}, Fe^{2+} and Fe^{3+} which contribute in improving agriculture productivity.

2.4.1 Agriculture

Humic acids have a big contribution in agriculture, particularly in soil fertility and plant nutrition. Humic acids able improve soil fertility by increase cation exchange capacity (CEC) in the soil (Harada & Inoko, 1975). It is a measurement of total capacity of a soil to hold exchangeable cations such as Fe^{3+} and Ca^{2+}. Thus, soils with large quantities of negative charge are more fertile because they retain more cations in their structure. This contributes to a better retention and utilization of minerals to the plant growth. Furthermore, humic acids increases the availability of nutrients in soil by lowering the pH of the soil to a more neutral level which will help to promote better plant health and growth. Besides luxuriant of plants, production or yield of the

agriculture product will increase greatly by increase of soil fertility as well. By increase the yield of agriculture product, food shortage around the world might be solved.

2.5 Ferrozine Assay

Ferrozine based colorimetric assay allow quantification of intracellular iron and determine of iron accumulation in cultured cells. Ferrozine is a ferroin compound widely used in determination of iron in biological samples. It is effective chelator of ferrous iron which forms a stable magenta-colored complex after chelatation with ferrous ion. Color change caused by chelate complex formulation of ferrozine and it will measured by spectrophotometer at 562 nm wavelength. The absorbance reading of this complex indicates the iron concentration in the solution. Complex forming is due to iron combined to transportation protein such as transferrin is denatured by weak acid and denaturant (Riemer, Hoepken, Czerwinska, Robinson, & Dringen, 2004). The principle of Ferrozine assay was shown in Figure 2.6.

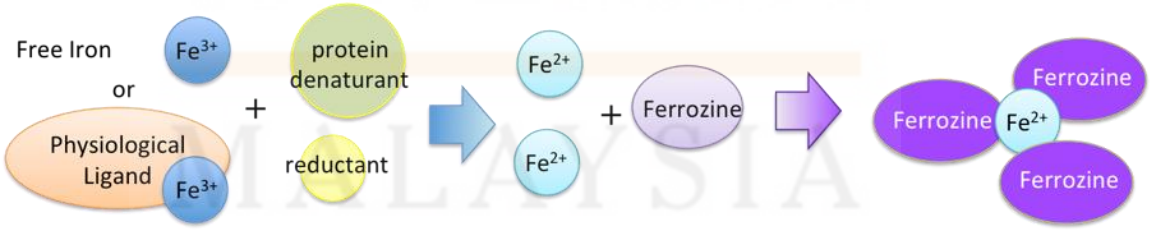


Figure 2.6: Ferrozine Assay Principle

CHAPTER 3

METHODOLOGY

3.1 Materials

The soil sampling required soil sampling bags, shovel and marker with water-proof ink.

The extraction of humic acid required soil samples, spatula, mortal, sodium hydroxide, hydrochloric acid, polyethylene centrifuge bottles, sieves, electronic balance, mechanical shaker, centrifuge, conical flask, dropping pipette and oven.

The LB agar plate preparation required LB agar powder, deionized water, electronic balance, autoclave, spatula, aluminum foil, schott bottle, measuring cylinder and beaker.

The LB broth preparation required LB broth powder, deionized water, electronic balance, autoclave, spatula, aluminum foil, schott bottle, measuring cylinder and beaker.

The humic acids solution preparation required dry humic acids, sodium hydroxide, mechanical shaker, falcon tube and beaker.

The preparation of ferrihydrite solution required iron (III) chloride hexahydrate, sterile distilled water, sodium hydroxide, hydrochloric acid, schott bottle, aluminum foil, spatula, electronic balance and pH meter.

The M1 media preparation required

1	Monopotassium phosphate (KH_2PO_4)
2	Dipotassium phosphate (K_2HPO_4)
3	Cobalt (II) sulphate heptahydrate ($COSO_4.7H_2O$)
4	Nickel (II) chloride hexahydrate ($NiCl_2.6H_2O$)
5	Sodium chloride (NaCl)
6	Boric acid (H_3BO_3)
7	Zinc sulphate heptahydrate ($ZnSO_4•7H_2O$)
8	Sodium molybdate dehydrate ($Na_2MoO_4•2H_2O$)
9	Copper (II) sulphate pentahydrate ($CuSO_4•5H_2O$)
10	Manganese (II) sulphate monohydrate ($MnSO_4•H_2O$)
11	Magnesium sulphate heptahydrate ($MgSO_4•7H2O$)
12	Calcium chloride dehydrate ($CaCl_2•2H_2O$)
13	Disodium salt (EDTA)
14	Iron (II) sulphate heptahydrate ($FeSO_4•7H_2O$)
15	Sodium selenite solution ($NaSeO_4$)
16	Sodium bicarbonate ($NaHCO_3$)
17	Ammonium sulphate ($(NH_4)_2SO_4$)

18	L-serine
19	L-arginine
20	L-glutamic acid
21	60% Na-Lactate solution

The cultivation of *S. oneidensis* MR-1 required LB agar, bunsen burner, incubator, autoclave and inoculum loop.

The growth of humic acids on microbial Fe (III) reduction activities required serum bottle, ferrihydrite solution, M1 media, humic acids solution, pipette, pipette tips, measuring cylinder, aluminum foil, bunsen burner, rubber stopper and caps.

The quantification of humic acid required spectrophotometer, spectrophotometric cuvettes, fourier-transform infrared spectroscopy, pipette and pipette tips.

The quantitation humic acids on microbial Fe (III) reduction activities required spectrophotometric cuvettes, pipette, pipette tips, needle, syringe, hydrochloric acid, spectrophotometer and ferrozine.

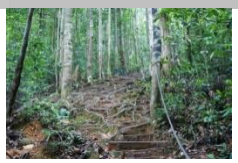
3.2 Methods

3.2.1 Soil Sampling

12 soil samples were collected from variety places as listed in Table 3.1. Each sample was loading into soil sampling bags with shovel and labeled with marker.

Table 3.1: 12 various soil sources with GPS location, collection date and local photos

Name	GPS Location	Collection Date	Local photos
Taman Orkid palm soil (TOP)	2°03'40.5''N 103°21'21.9''E	11 September 2018	
Kelantan palm soil (PK)	5°90'80.3''N 101°93'30.7''E	4 October 2018	
Kampung Sembrong palm soil (KSP)	2°03'40.5''N 103°21'21.9''E	11 September 2018	
Kampung Baru Kluang palm soil (KBKP)	2°03'58.9''N 103°20'47.4''E	11 September 2018	
Kampung Melayu palm soil (KMP)	2°02'56.1''N 103°19'30.3''E	11 September 2018	

Kampung Tengah Indah palm soil (KTIP)	2°03'55.1''N 103°20'38.7''E	11 September 2018	
Kampung Tengah Indah pinapple soil (KTP)	2°03'51.7''N 103°20'25.4''E	11 September 2018	
Gunung Lambak forest soil (GLFS)	2°01'84.0''N 103° 20' 00.4''E	11 September 2018	
Perak paddy soil (PP)	4°02'35.0''N 101°10'23.0''E	25 October 2018	
Sekinchan paddy soil (PS)	3°29' 59.9'' N 101°05' 60.0'' E	28 October 2018	
Agropark vegetable garden soil (VA)	5°44' 46.9'' N 101°52' 08.1'' E	17 October 2018	
Kluang vegetable garden soil (VK)	2°01'49.3''N 103°19'46.8''E	11 September 2018	

3.2.2 Extraction of Humic Acids

After collection, soil samples were sieved with 2 mm mesh. 5 g of sample from different soil sources was placed into conical flask by following adding of 50 mL of 0.5M NaOH solution into it. The flasks were put in mechanical shaker for 24 hours. After 24 hours, side of the bottles were washed using distilled water and samples were centrifuged once at 10,000 r.p.m for 15 minutes. The dark color of supernatant liquors was decanted and pH of solutions was adjusted to 1.0 with 6M of HCl. The solution was equilibrated at room temperature for 8 hours. 2 layers of solution were formed and the supernatants were siphoned off. The remainder suspensions were transferred to polyethylene bottles and it was suspended in 50 mL distilled water followed by centrifuged at 10,000 r.p.m for 10 minutes. The supernatants were decanted and this procedure was repeated 3 times. The washed humic acids were dried in oven at 40 ^{o}C to a constant weight (Chang, 2001).

3.2.3 Luria (LB) Agar Medium Preparation (1L)

40 g of LB agar powder was dissolve in 1000 mL deionized water. Solution was autoclave before used and allows cooling before poured to the petri dish.

3.2.4 Luria (LB) Broth Medium Preparation (1L)

25g of LB broth powder was dissolve in 1000mL deionized water. 5 mL of the solution was equally poured into test tube with cap. The broth was autoclave before used.

3.2.5 Preparation of Humic Acids Solution

Dried humic acids were dissolved in 0.1 M NaOH and placed on mechanical shaker overnight to completely dissolve the humic acids in the solution. The solution was autoclaved before use (B. Libecki, 2007).

3.2.6 Preparation of Ferrihydrite

0.4 M FeOOH stock solution was prepared by added 54.06 g of $FeCl_3 \cdot 6H_2O$ into 500 mL sterile dH_2O. The pH of the solution was adjusted to pH 7 by using 10N NaOH. The solution was then stored in the dark.

3.2.7 Preparation of M1 Media

8 solutions were prior-prepared to make minimal medium M1 for *S. oneidensis* MR-1. They were phosphate buffer, metal supplement, trace element solution, basal salts, mixed amino acids, 30% Na-Lactate, 115mM sodium selenite ($NaSeO_4$), 200 mM sodium bicarbonate ($NaHCO_3$).

Phosphate buffer	Mixed 30.0 g of monopotassium phosphate (KH_2PO_4) and 86.1 g of dipotassium phosphate (K_2HPO_4) into 800 mL distilled water. The solution was adjusted to pH 7 and the final volume was bringing to 1 litre by adding distilled water.
Metal supplement solution	1.41 g of cobalt (II) sulphate heptahydrate ($COSO_4.7H_2O$), 1.98 g of nickel (II) chloride hexahydrate ($NiCl_2.6H_2O$) and 0.58 g of sodium chloride (NaCl) was added into 100 mL of distilled water. The solution was autoclave before used and stored in dark.
Trace element solution	Mixed 2.8 g of boric acid (H_3BO_3), 0.24 g of zinc sulphate heptahydrate ($ZnSO_4•7H_2O$), 0.75 g of sodium molybdate dihydrate ($Na_2MoO_4•2H_2O$), 0.042 g of copper (II) sulphate pentahydrate ($CuSO_4•5H_2O$) and 0.17 g of manganese (II) sulphate monohydrate ($MnSO_4•H_2O$) in 1 litre distilled water. The solution was stored at 4 ^oC.
Basal salts solution	Added 10 mL of trace element solution, 2.0 g of magnesium sulphate heptahydrate ($MgSO_4•7H2O$), 0.57 g of calcium chloride

	dihydrate ($CaCl_2$•$2H_2O$), 0.20 g of disodium salt (EDTA) and 0.012 g of iron (II) sulphate heptahydrate ($FeSO_4$•$7H_2O$) into 800 mL of distilled water. The solution was stored at 4 ^oC.
Mixed amino acids solution	Added 0.2 g each of L-serine, L-arginine and L-glutamic acid into 100 mL of distilled water. The solution was autoclave before used and stored at 4 ^oC.
30% Na-Lactate solution	Mixed 200 mL of 60% Na-Lactate solution with 200 mL of distilled water
115 mM sodium selenite solution ($NaSeO_4$)	Added 1.086 g of sodium selenite ($NaSeO_4$) into 50 mL of distilled water.
200 mM sodium bicarbonate solution ($NaHCO_3$)	Added 4.2 g of sodium bicarbonate ($NaHCO_3$) into 250 mL of distilled water.

After preparing well all the solution required, 0.95 g of ammonium sulphate ($(NH_4)_2SO_4$) was added to a schott bottle to prepare 500 mL of minimal medium M1. Then, 7.5 mL of phosphate buffer, 50 mL of basal salts solution and 438 mL of distilled water were poured and mixed well in the bottle. Next, 0.05 mL each of 115 mM sodium selenite solution ($NaSeO_4$) and metal supplement were added into the bottle followed by 6 mL of 30% Na-Lactate solution. The minimal medium M1 was adjusted to pH 7 by 10N NaOH and the solution was autoclaved before used.

3.2.8 Bacteria Strain and Cultivation Condition

S. oneidensis MR-1 was grown on LB agar plate and incubated overnight at 30 °C. A single colony of bacteria was then inoculated into 5 mL of LB broth and cultured in orbital shaker overnight at 30 °C to serve as a seed culture. The seed culture was then transfered to a 50 mL of LB medium. The medium was cultured until the OD_{600} was between 0.8-1.2 and was ready for microbial Fe(III) reduction activities.

3.2.9 Cultivation Humic Acids on Microbial Fe(III) Reduction Activities

In this study, 2 controls were been carried out to determine the effectiveness of humic acids on microbial Fe(III) reduction as an electron shuttle. There were control which the M1 medium consist of *S. oneidensis* MR-1 cell suspension and ferrihydrite solution under anaerobic condition whereas zero which the M1 medium only consist of ferrihydrite solution under anaerobic condition.

To prepare 26 serum bottles (120 mL), 2340 mL of M1 media with 260 mL of HFO solution (9 M1 media : 1 HFO) was mixed together. 100 mL of mixed solution was then poured equally to each bottle. To compare the concentration of humic acids with microbial Fe(III) reduction, a humic acids stock solution was prepared in 0.25 g/10 mL. Difference concentration of humic acids solution was pipetted into bottles based on concentration: 0 mg/L (0µl), 5 mg/L (18 µl), 10mg/L (36 µl), 25 mg/L (90 µl), 50 mg/L (180 µl) and 100 mg/L (360 µl). To carry out microbial Fe (III) reduction, *S. oneidensis*

MR-1 which had OD_{600} = 0.8~1.2 was centrifuged at 6000 r.p.m in 10 minutes. The supernatant was decanted and the pellet was resuspended in M1 media. Absorbance reading was recorded and measured by spectrophotometer at 600 nm wavelength before and after the bacteria culture poured into the serum bottles (0 hour). The reading of bacteria culture should have OD_{600} between 0.8-1.2. 2000 µl of bacteria culture was pipetted into each bottle. All the bottles were sealed with rubber stopper and cap. Nitrogen gas was pumped into the bottle around 5 minutes per bottles by needle. The bottles were stored in dark.

3.2.10 Quantification of Humic Acids

12 various extracted humic acids were determined by spectrophotometer at wavelength λ = 465 and 665 nm (L.T. Shirshova *et al*., 2015) and Fourier-transform infrared spectroscopy (FTIR).

3.2.11 Quantitation Humic Acids on Microbial Fe(III) Reduction Activities

Each of the control and culture were tested for Fe(III) reduction activities by using Ferrozine assay test. Briefly, 1000 µl syringe and needle were used to take the sample from each of the bottle. 200 µl of the sample was taken out and transferred to 1000 µl of 0.5N HCl in a mircocentrifuge tube and kept in dark around 10-15 minutes. To quantify concentration of Fe(II), 950 µl of ferrozine was added into new

microcentrifuge tubes with 50 µl of sample. The absorbance of the complex was measured within 10-15 minutes at 562 nm by using Thermo Spectronic GeneSys 20 spectrophotometer.

Fe(II) concentration could be calculated from the final absorbance reading of a sample (Wee, 2014).

$$\text{Fe(II) concentration (mM)} = \frac{(final\ reading - 0.016)}{0.199}$$

CHAPTER 4

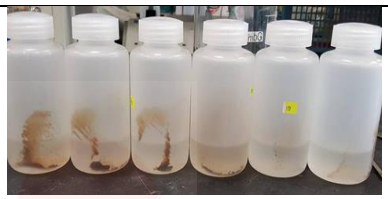
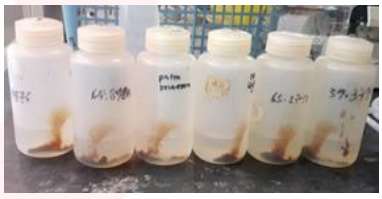
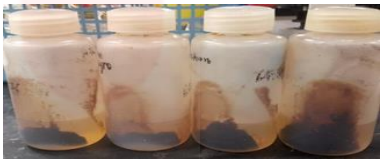
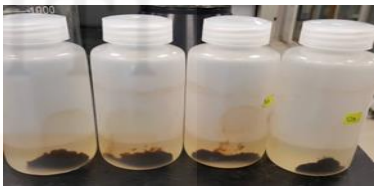
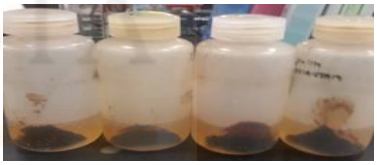
RESULT AND DISCUSSIONS

4.1 Extraction Humic Acids

Humic acids were extracted from 12 different soil sources. Percentage of recovery was calculated to determine the relationship between the effectiveness of humic acids on microbial Fe(III) reduction with the recovery (%).

Table 4.1: Extracted humic acids from 12 various soil sources with recovery (%) and pictures

Name	Recovery (%)	Pictures
Taman Orkid palm soil (TOP)	$\frac{0.4238}{30} x100 = 1.413$	

Kelantan palm soil (PK)	$\frac{0.1394}{30} x100 = 0.465$	
Kampung Sembrong palm soil (KSP)	$\frac{0.3513}{30} x100 = 1.171$	
Kampung Baru Kluang palm soil (KBKP)	$\frac{0.5602}{30} x100 = 1.867$	
Kampung Melayu palm soil (KMP)	$\frac{0.2468}{30} x100 = 0.823$	
Kampung Tengah Indah palm soil (KTIP)	$\frac{1.1492}{30}$ x100 = 3.831	
Kampung Tengah Indah pinapple soil (KTP)	$\frac{0.7577}{30} x100 = 2.526$	
Gunung Lambak forest soil (GLFS)	$\frac{0.3361}{30} x100 = 1.120$	

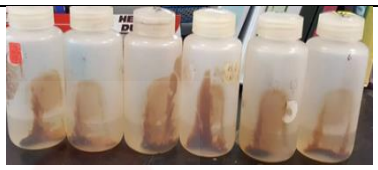
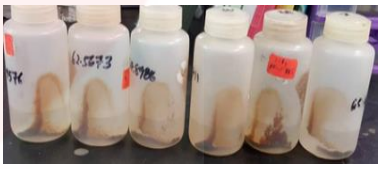
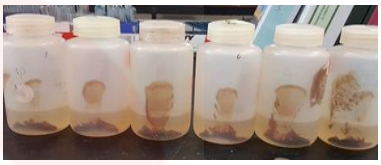
Perak paddy soil (PP)	$\frac{0.2317}{30} x100 = 0.772$	
Sekinchan paddy soil (PS)	$\frac{0.2756}{30} x100 = 0.919$	
Agropark vegetable garden soil (AG)	$\frac{0.052}{30} x100 = 0.173$	
Kluang vegetable garden soil (VK)	$\frac{0.1628}{30} x100 = 0.543$	

Table 4.1 illustrated the extracted humic acids from 12 various soil sources. Each type of soil source yield different amount of humic acids from 30 g of soil samples. Properties of humic acids are depend on vary characteristics such as climate, soil source and others. Hence, different origins of soil give different properties of humic acids such as amount, quality and color.

4.2 Determination of *S. oneidensis* MR-1 Growth Curve

When fresh liquid medium is inoculated with a given number of bacteria and incubated for sufficient period of time, it gives a characteristic growth pattern of bacteria. In this experiment, *S. oneidensis* MR-1 was inoculated in LB agar at 30 ^{o}C for 24 hours.

The growth curve of *S. oneidensis* MR-1 was determined by observing their growth in LB broth at room temperature for 35 hours.

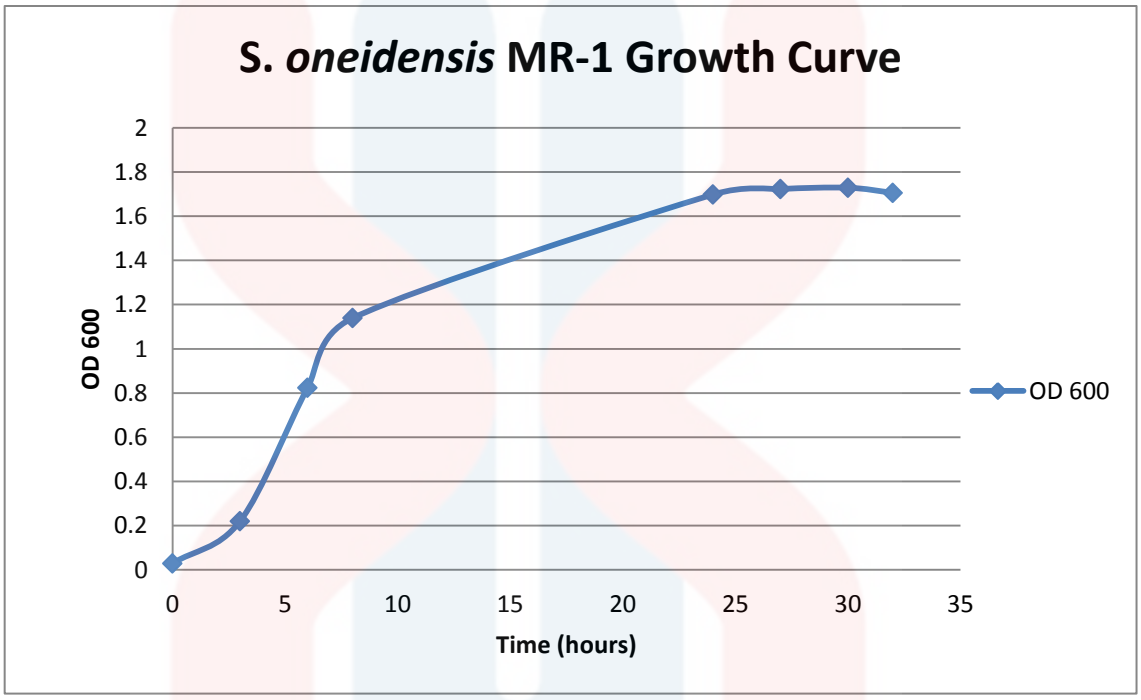


Figure 4.1: Growth curve of *S. oneidensis* MR-1 with time from 0 to 35 hours.

The growth curve of bacteria showed hyperbolic graph where the bacteria undergo lag phase from 0 to 3 hours. Then, the bacteria were exponentially its population from 6 to 24 hours which known as log phase. In this phase, the bacteria were in a rapidly growing and dividing state. After that, the bacteria were undergo stationary phase from 27 to 30 hours where the bacteria growth reaches a state during which there is no net increase in bacterial population. Lastly, the numbers of bacteria started decrease continuously from 32 hours which called as death phase. During this phase, the bacteria completely lose its ability to reproduce which might due to the depletion of nutrients and the subsequent accumulation of metabolic waste products and other toxic materials in

the media. From the curve, bacteria concentration of *S. oneidensis* MR-1 which having OD_{600} 0.8~1.2 had been chosen to carry out microbial Fe(III) reduction.

4.3 Quantification of Microbial Fe(III) Reduction Activities via Ferrozine Assay

Abiotic and biotic microbial Fe(III) reduction with different humic acids concentration was carried out to compare the production of Fe(II) concentration. Ferrozine assay was executed to quantified Fe(II) concentration.

Figure 4.2 demonstrated that abiotic and biotic microbial Fe(III) reduction of extracted humic acids from Kampung Tengah Indah pinapple soil (KTP) in different humic acids concentration (0 mg/L, 5 mg/L, 10 mg/L, 25 mg/L, 50 mg/L, 100 mg/L). *S.oneidensis* MR-1 served as Fe(III) reducing bacteria which enable used Fe(III) as electron acceptor in anaerobic condition. This was been proven that the entire biotic medium had high Fe(III) reduction rate compared to abiotic medium. Thus, *S.oneidensis* MR-1 plays an important part in Fe(III) reduction.

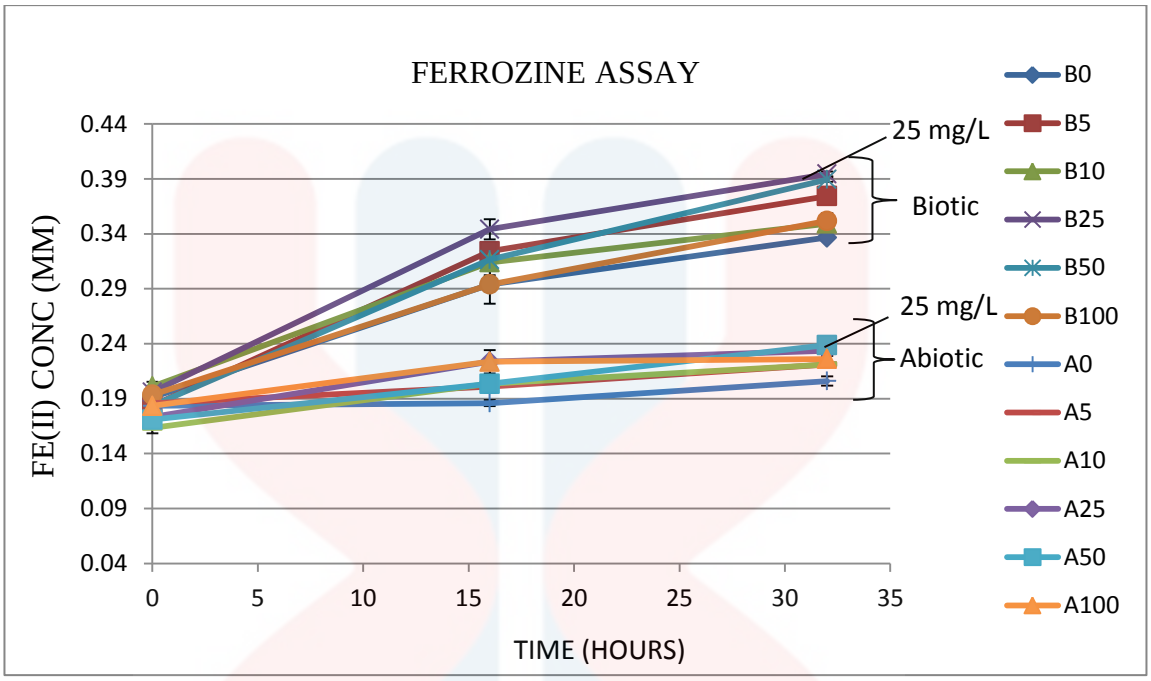


Figure 4.2: Abiotic (A) and biotic (B) microbial Fe(III) reduction with different concentration of extracted humic acids from Kampung Tengah Indah pinapple soil (KTP), control and zero with lactate as the sole electron donor and Fe(III) (HFO) as the sole electron acceptor in anaerobic condition. Fe(III) reductions were representing by the production of Fe(II) via ferrozine assay. Error bars indicate standard deviation.

Among 6 various concentrations of extracted humic acids on microbial Fe(III) reduction activities, 25 mg/L shown the supreme reduction rate compared to other concentrations. In contrast, medium utilized of higher concentration of extracted humic acids such as 50 mg/L and 100 mg/L did not shown obvious reduction rate on microbial Fe(III) reduction. Thus, the higher concentration of extracted humic acids used on microbial Fe(III) reduction did not offered an outstanding result on Fe(III) reduction. 25 mg/L of extracted humic acids stock solution was chosen for further microbial Fe(III) activities.

Moreover, medium cultivated in anaerobic condition shown higher Fe(II) concentration compared to aerobic condition. This proven that Fe(II) only stable in acidic condition under aerobic condition and it is rapidly oxidized to Fe(III) at neutral pH.

To compare the different properties of humic acids on microbial Fe(III) reduction, 12 various humic acids from different soil sources were tested anaerobically in minimal growth medium (M1 media) with lactate as the sole electron donor and Fe(III) (HFO) as the sole electron acceptor. FTIR was then used to define the functional group of humic acids to determine the relationship between microbial Fe(III) reductions with the humic acid's functional group. Ferrozine assay was executed to quantify the concentration of Fe(II) in the solution. The quantification of Fe(III) reduction was shown in Figure 4.3.

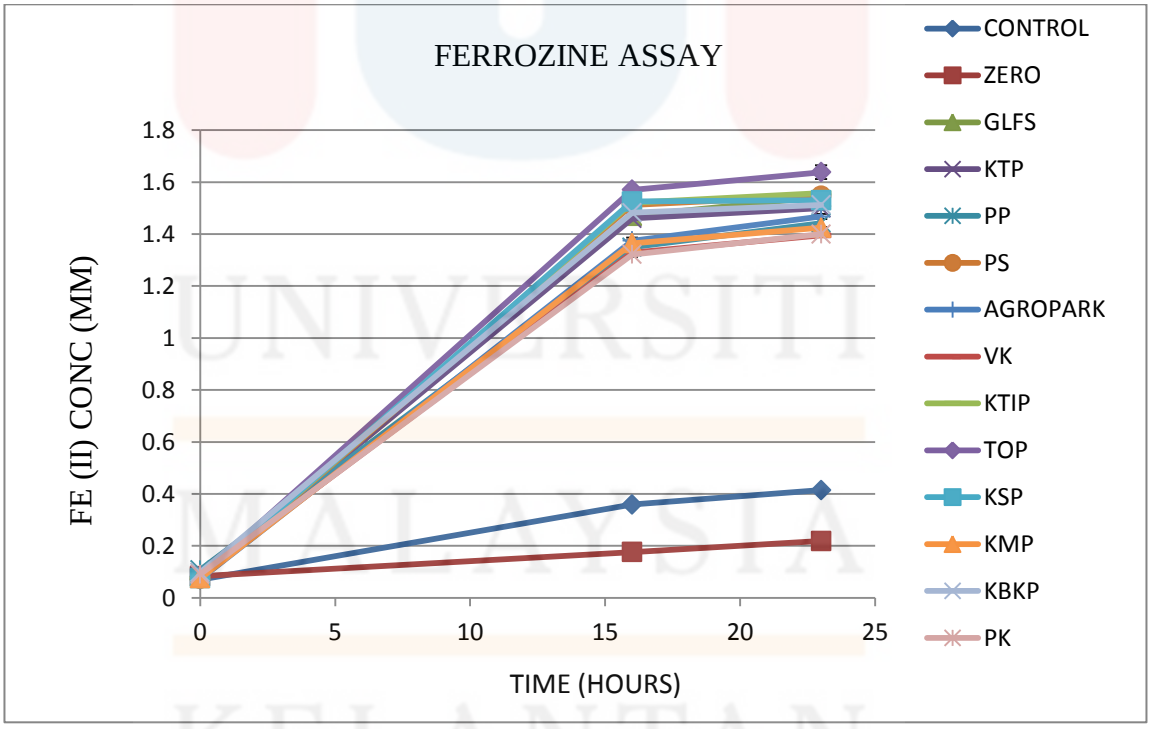


Figure 4.3: Fe(III) reduction by 12 type of extracted humic acids from variety soil sources, control and zero with lactate as the sole electron donor and Fe(III) (HFO) as the

sole electron acceptor in anaerobic condition. Fe(III) reductions were representing by the production of Fe(II) via ferrozine assay. Error bars indicate standard deviation.

Figure 4.3 showed that all of the extracted humic acids are able reduce Fe(III) to Fe(II) with lactate as sole electron donor and HFO as electron acceptor. TOP illustrated highest Fe(II) production, followed by KTIP, PS, GLFS, KSP, KBKP, KTP, VA, PP, KMP, PK, VK, control and zero.

Medium with additional humic acids showed higher Fe(II) production than control and zero. This proven the effectiveness of humic acids acts an electron shuttle in Fe(III) reduction. By employing the electron shuttle, the rate of electron transfer will be increase. According to Kato (2016), electron shuttle compounds such as humic acids or AQDS have the properties of redox potential. The redox potential means a compound has reducing or oxidizing capacity. Potential used of this electron shuttle compounds help enhance and maintain the nutrients and iron bioavailability in soil. The *S. oneidensis* MR-1 will reduce Fe(III) bound in the humic acids during the reduction process. Microorganisms have more electronegative potential than the electron shuttle compounds. Thus, electron shuttle compounds will accept the electrons from the microorganism and transfer it to reduce the Fe(III). After transferring, the electron shuttle compounds will regenerate to its oxidized form as respiratory substrates.

Ferrozine reagent was adopted to quantify concentration of Fe(II) in the solution which was achieved by measuring the absorbency of the ferrous-ferrozine complex at 562 nm. Purple colored complex was formed when the ferrozine reagent reacted with the ferrous iron in the solution. The absorbance is directly proportional to the amount of iron

in the solution. The darker the purple color, the higher presence of Fe(II) concentration in the solution.

Before ferrozine assay, samples were acidified by 0.5 M hydrochloric acid (HCl) which used to stabilize the Fe(II) during the analysis. It is well known that Fe(II) is comparatively stable in acid solution and that it is rapidly oxidized to the Fe(III) by the oxygen of the air in alkaline solution. This is because hydrogen from the acid is reacts with the oxygen in the air. Thus, prevent the oxygen from reacting with the iron. It's easier for the oxygen to bind with hydrogen than with iron.

To determine the effect of humic aicds on microbial growth, *S.oneidensis* MR-1 was cultured on M1 medium aerobically.

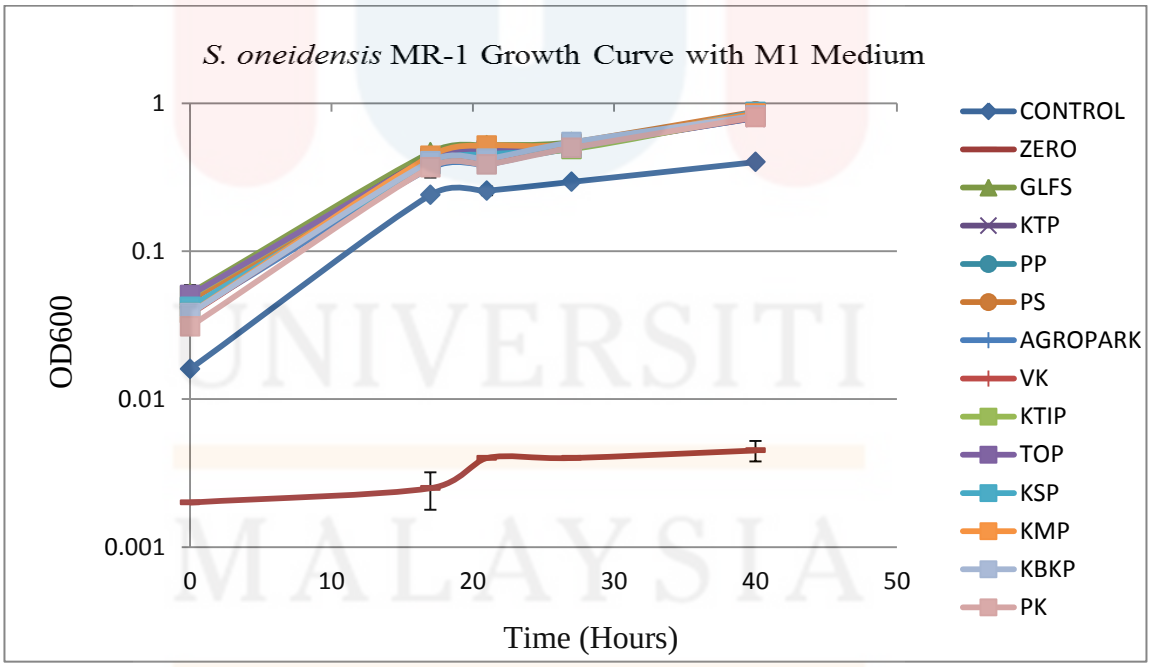


Figure 4.4: *S. oneidensis* MR-1 growth curve by 12 types of extracted humic acids from various soil sources, control and zero with M1 medium as the growing medium in aerobic condition. Error bars indicate standard deviation.

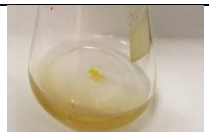
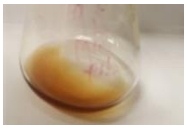
Figure 4.4 represented that humic acids able contributed nutrient to the *S. oneidensis* MR-1 growth in aerobic condition. This had been proven from Figure 4.4 which growth rate of the bacteria with humic acids addition significantly shown higher rate compared to control and zero.

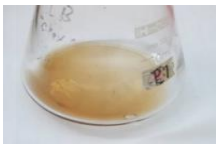
4.4 Quantification of Humic Acids

4.4.1 Spectrophotometer

The E4/E6 ratio is implied to the degree of condensation and aromaticity of the humic substances and to their degree of humification. Thus, distinct types of extracted humic acids had shown various E4/E6 ratio values.

Table 4.2: 12 various humic acids solution in OD_{465}, OD_{665}, E4/E6 ratio and pictures

Soil Type	465nm	665nm	E4/E6 ratio	
Agropark vegetable garden soil (AG)	0.383	0.103	3.718	
Sekinchan paddy soil (PS)	1.903	0.217	8.770	

Perak paddy soil (PP)	0.876	0.214	4.093	
Kluang vegetable garden soil (VK)	3.228	1.390	2.322	
Taman Orkid palm soil (TOP)	1.273	0.171	7.44	
Kelantan palm soil (PK)	0.239	0.090	2.656	
Kampung Sembrong palm soil (KSP)	0.783	1.106	7.387	
Kampung Baru Kluang palm soil (KBKP)	1.088	0.122	8.918	
Kampung Melayu palm soil (KMP)	0.587	0.142	4.134	
Kampung Tengah Indah pinapple soil (KTP)	0.623	0.091	6.846	
Kampung Tengah Indah palm soil (KTIP)	1.802	0.209	8.622	

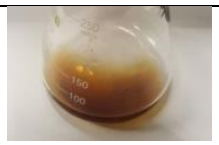
Gunung Lambak forest soil (GLFS)	0.909	0.129	7.047	

Table 4.2 showed the humic acids which extracted from 12 various soil sources with it's OD_{465}, OD_{665} and E4/E6 ratio.

Humic acids exracted from KBKP showed a remarkable high value than other 11 extracted humic acids which E4/E6 ratio = 8.918, followed by PS, KTIP, TOP, KSP, GLFS, KTP, KMP, PP, AG, PK and VK.

From the observation, higher E4/E6 ratio of the extracted humic acids, the darker of it color. According to K. Kumada (1965), they might essentially high molecular amorphous heteropolycondensates of brown or black colour. The dark colour is principally due to the existence of numerous and various kinds of conjugated double bonds which are randomly distributed in the molecules. Different components consists in the various sources of humic acids showed different reaction in microbial Fe(III) reduction.

4.4.2 Fourier-Transform Infrared Spectroscopy (FTIR)

To determine the relationship of humic acids functional group with its effect on microbial Fe(III) reduction, FTIR should be carrying out. 6 different types of humic acids were selected and study based on their microbial Fe(III) reduction. The prepared

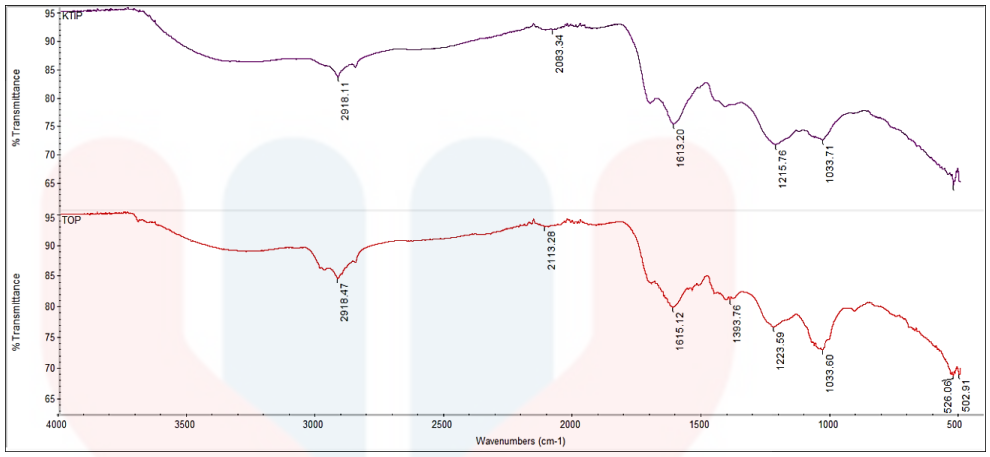
samples were analyzed by FTIR at wavenumber region of 400-4000cm^{-1} to determine the chemical structure. The vibration is summarized in Table 4.3.

Table 4.3: Relative intensity of major IR absorption bands of humic acids

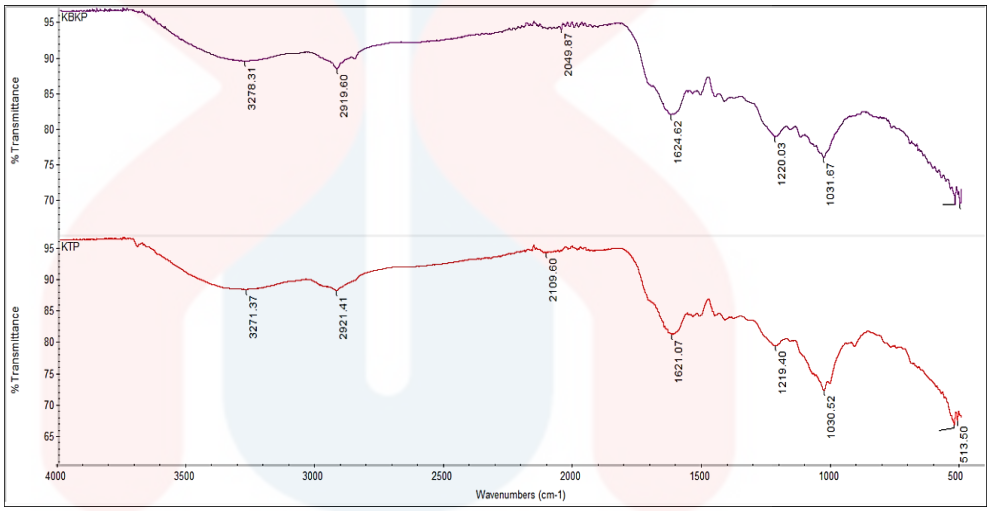
Wavelength (cm^{-}1)	Assignment
3692-3229	H-bonded OH, Free OH
2987-2083	Aliphatic C-H
1636-1621	C=O stretching of amide group, carboxylic acids, aldehydes, ketones and quinones
1620-1600	Aromatic C=C, strongly H bonded C=O of conjugated ketones
1582	COO^- symmetric stretching, N-H bending, C=N stretching
1393	OH deformation, C-O stretching of phenolic OH, COO^- antisymmetric stretching
1228-1215	C-O stretching and OH deformation of COOH
1031-1003	C-O of alcohol, ether, polysaccharide group and O-H of alcohol
911-502	Al-O-Si

(Source: (G. Matrajt, 2004; M.A. Mohamed, 2017; Orsetti, Quiroga Mde, & Andrade, 2006; Ummo Fooken 2003)

(a)



(b)



(c)

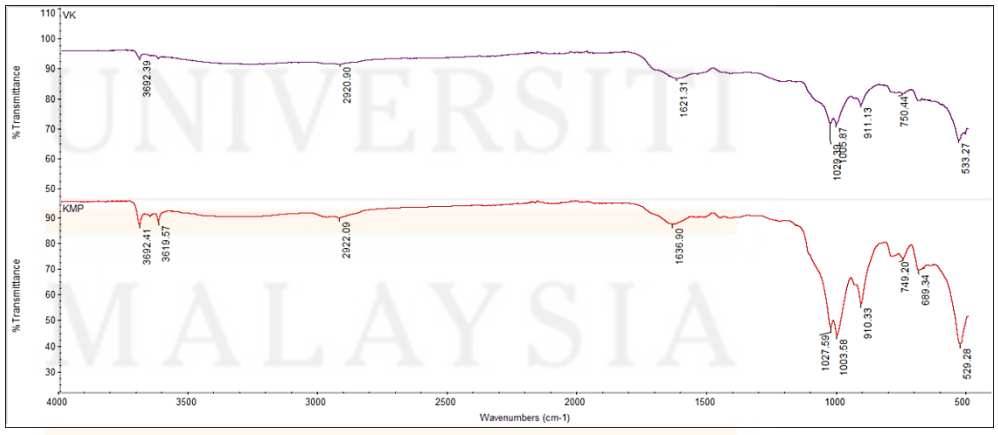


Figure 4.5 (a) FTIR spectrum of extracted humic acids from Taman Orkid palm soil (TOP) and Kampung Tengah Indah palm soil (KTIP), (b) FTIR spectrum of extracted humic acids from Kampung Baru Kluang palm soil (KBKP) amd Kampung Tengah

Indah pinapple soil (KTP), (c) FTIR spectrum of extracted humic acids from Kampung Melayu palm soil (KMP) and Kluang vegetable garden soil (VK)

To better understand the properties of extracted humic acids on the effect of endogeneous humic acids on microbial Fe(III) reduction. 6 different types of extracted humic acids were selected from previous microbial Fe(III) reduction activity to carry out fourier-transform infrared spectroscopy (FTIR). They were divided into 3 different groups based on their Fe(III) reduction ability. There were humic acids which had shown high rate on Fe(III) reduction: Taman Orkid palm soil (TOP) and Kampung Tengah Indah palm soil (KTIP). Next, humic acids which shown moderate Fe(III) reduction rate: Kampung Baru Kluang palm soil (KBKP) amd Kampung Tengah Indah pinapple soil (KTP). Last, humic acids which shown low Fe (III) reduction rate: Kampung Melayu palm soil (KMP) and Kluang vegetation soil (VK).

Based on the table of characteristic of IR absorptions, functional group at different peak can be determined. A broad band at 2083-2918 cm^{-1} found in figure 4.5 (a) corresponding to aliphatic C-H; a peak at 1613-1615 cm^{-1} illustrated aromatic C=C; a band at 1393 cm^{-1} found in extracted humic acids of Taman Orkid palm soil (TOP) indicated OH of phenol and alcohol group; a broad band at 1215-1223 cm^{-1} produced mainly by C-O and O-H of COOH; the second less marked shoulder at 1033 cm^{-1} characterizes C-O of alcohol, ether, polysaccharide groups and OH of alcohol, and a weak band exhibit in extracted humic acids of Taman Orkid palm soil (TOP) at 502-526 cm^{-1} corresponding to Al-O-Si.

Figure 4.5 (b) showed the IR spectra of Kampung Baru Kluang palm soil (KBKP) amd Kampung Tengah Indah pinapple soil (KTP). A weak band at 3278-3271 cm^{-1} corresponding to OH stretching; a peak at 2919-2921 cm^{-1} produced mainly by aliphatic C-H; a broad band between 1621-1624 cm^{-1} corresponding to stretching mode C=C of ketone; a less intense band between 1219-1220 cm^{-1} characterized C-O and O-H of COOH, a peak at 1030-1031 cm^{-1} produced mainly by C-O of alcohol, ether, polysaccharide groups and OH of alcohol, and a weak band at 513 cm^{-1} presence in extracted humic acids of Kampung Tengah Indah pinapple soil (KTP) corresponding to Al-O-Si.

Figure 4.5 (c) showed a typical IR spectrum obtained from the Kampung Melayu palm soil (KMP) and Kluang vegetation soil (VK) in the 400−4000 cm^{-1} wavelength. A weak band at 3619-3692 cm^{-1} corresponding to OH stretching; a peak at 2920-2922 cm^{-1} produced mainly by aliphatic OH stretching; a less intense band at 1636 cm^{-1} exist in IR spectra of Kampung Melayu palm soil (KMP) characterizes aromatic C=C,C=O in amide (I), ketone and quinone group; a less intense band at 1621 cm^{-1} exist in IR spectra of Kluang vegetation soil (VK) characterizes stretching mode C=C of ketone; a broad band between 1003-1029 cm^{-1} produced mainly by C-O of alcohol, ether, polysaccharide groups and OH of alcohol, and peak between 911-529 cm^{-1} corresponding to Al-O-Si.

Based on the result showed in the Figure 4.5, functional groups existed among 6 types of humic acids is not much divergence by comparison of their IR spectrum. However, they showed distinct effect on microbial Fe(III) reduction. This might due to diverse of bond's intensity presence in various humic acids.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The study was carried out to extract 12 various types of humic acids from different soil sources which are Taman Orkid palm soil (TOP), Kampung Tengah Indah palm soil (KTIP), Sekinchan paddy soil (PS), Gunung Lambak forest soil (GLFS), Kampung Sembrong palm soil (KSP), Kampung Baru Kluang palm soil (KBKP), Kampung Tengah Indah pinapple soil (KTP), Agropark vegetarian soil (VA), Perak paddy soil (PP), Kampung Melayu palm soil (KMP), Kelantan palm soil (PK) and Kluang vegetation soil (VK). With dissimilatory metal-reducing bacterium, *Shewanella oneidensis* MR-1, all of the extracted humic acids showed their ability on microbial Fe(III) reduction with lactate as the sole electron donor and Fe(III) (HFO) as the sole electron acceptor in anaerobic condition. Microbial Fe (III) reduction of extracted humic acids from Taman Orkid palm soil (TOP) displayed the highest Fe(III) reduction

compared to other extracted humic acids. The additional of extracted humic acids in the media showed enhancement in the Fe(III) reduction which proven the statement that humic acids serve as electron shuttle in Fe(III) reduction. Fourier-transform infrared spectroscopy and spectrophotometer were applied to characterize various types of extracted humic acids.

5.2 RECOMMENDATIONS

In this study, the effect of endogeneous humic acids on microbial Fe(III) reduction was investigated. To characterize various types of the endogeneous humic acids, it is suggested to used anthraquinone-2,6-disulfonate (AQDS) as a control. AQDS have been used as an artificial electron shuttle as substitute for humic acids.

Many bacterial species have been identified that utilize insoluble metal oxides for respiration. To determine the variety of respiratory capabilities of bacteria on Fe(III) reduction, soluble Fe(III) solution (FeCi) could be used to determine the effect of endogeneous humic acids on microbial Fe (III) reduction.

REFERENCES

Aranda, V., & Comino, F. (2014). Soil organic matter quality in three Med iterranean environments (a first barrier against desertification in Europe). J. Soil Sci. Plant Nutr. 2014, vol.14, n.3, pp.743-760. Epub 02-Ago-2014. ISSN 0718-9516. http://dx.doi.org/10.4067/S0718-95162014005000060

B. Libecki, J. D. (2007). Optimization of Humic Acids Coagulation with Aluminum and Iron(III) Salts.

Baskakov, S. A., Lobach, A. S., Vasil'ev, S. G., Dremova, N. N., Martynenko, V. M., Arbuzov, A. A., . . . Shul'ga, Y. M. (2016). High-temperature carbonization of humic acids and a composite of humic acids with graphene oxide. *High Energy Chemistry, 50*(1), 43-50. doi: 10.1134/s0018143916010021

Bleam, W. (2017). Natural Organic Matter. Soil and Environmental Chemistry.2017, pp. 333-384. doi: 10.1016/b978-0-12-804178-9.00007-0

Bocanegra, M. P., Lobartini, J. C., & Orioli, G. A. (2006). Plant Uptake of Iron Chelated by Humic Acids of Different Molecular Weights. *Communications in Soil Science and Plant Analysis, 37*(1-2), 239-248. doi: 10.1080/00103620500408779

Brondi, Ariadne Missono Daniel, Josiane Souza Pereira Castro, Vitor Xavier Monteiro de Bertoli, Alexandre Carvalho Garcia, Jerusa Simone Trevisan, Marcello Garcia. (2016). Quantification of Humic and Fulvic Acids, Macro- and MicroNutrients and C/N Ratio in Organic Fertilizers. Communications in Soil Science and Plant Analysis. 2506-2513. 10.1080/00103624.2016.1254791

Bucking, C., Piepenbrock, A., Kappler, A., & Gescher, J. (2012). Outer-membrane cytochrome-independent reduction of extracellular electron acceptors in

Shewanella oneidensis. *Microbiology, 158*(Pt 8), 2144-2157. doi: 10.1099/mic.0.058404-0

Cassandra Worman and Julia McMorrow. (2002). Acrotelm and Catotelm. Retrieved from http://media.human ities.manchester.ac.uk/huma nities/geog_dark_peak/ac rotelm.htm.

Chang, M. C. W. a. S. H. (2001). Mean residence times and characteristics of humic substances extracted from a Taiwan soil. Canadian Journal of Soil Science. 2001, 81(3): 299-307, https://doi.org/10.4141/S00-068

Derek R. Lovley, J. D. C., Elizabeth L. Blunt-Harris, Elizabeth J. P. Phillips and John C. Woodward. (1996). Humic substances as electron acceptors for microbial respiration. Nature volume 382, pages 445–448 (01 August 1996), https://doi.or g/10.1038/382445a0

Fenchel, T., King, G. M., & Blackburn, T. H. (2012). *Degradation of Organic Polymers and Hydrocarbons*. Bacterial Biogeochemistry (Third Edition). 2012, pp. 49-57, https://doi.org/10.1016/B978-0-12-415836-8.00003-7

Friedhelm G, l., Gerhard Langenberger and Peter Widmann (2006). Tropical Lowland Evergreen Rainforest. Ecology of Insular Southeast Asia. 2006, 297–383. doi:10.1016/b978-044452739-4/50017-6

Gyana R. Rout and Sunita Sahoo. (2015). Role of Iron in Plant Growth and Metabolism. Reviews in Agricultural Science, 3: 1-24, 2015.doi: 10.7831/ras.3.1

G. Matrajt, J.Borg,P.I.Raynal, Z. Djouadi, L. d'Hendecourt, G. Flynn, and D. Deboffl. (2004). FTIR and Raman analyses of the Tagish Lake meteorite: Relationship with the aliphatic hydrocarbons observed in the Diffuse Interstellar Medium. Worldwide astronomical and astrophysical research. 2004, Vol. 416, Number 3, pp. 983 - 990, doi: 10.1051/0004-6361:20034526

Gralnick, N. J. K. J. A. (2013). Flavin Electron Shuttles Dominate Extracellular Electron Transfer by Shewanella oneidensis. MBio, *4*(1). doi: 10.1128/mBio.0055312

Harada, Y., & Inoko, A. (1975). Cation-exchange properties of soil organic matter. Soil Science and Plant Nutrition, 21(4), 361-369. doi: 10.1080/00380768.1975.10432651

J. A. Srnnms, E.A. Paul, R. J. Sr. Arnaud and W. K. Head. (1968). Spectrophotometric Measurement of Soil Color and its Relationship to moisture and organic matter.

Kasai, T., Kouzuma, A., & Watanabe, K. (2017). CRP Regulates D-Lactate Oxidation in Shewanella oneidensis MR-1. Frontiers in Microbiology, 8(869). doi: 10.3389/fmicb.2017.00869

Kato, S. (2016). Microbial extracellular electron transfer and its relevance to iron corrosion. *Microb Biotechnol, 9*(2), 141-148. doi: 10.1111/1751-7915.12340

Kumada, K. (1965). Studies on the colour of humic acids. *Soil Science and Plant Nutrition, 11*(4), 11-16. doi: 10.1080/00380768.1965.10433805

Kumada, K. (1987). Chapter 3 Spectroscopic Characteristics of Humic Acids and Fulvic Acids. Chemistry of Soil Organic Matter. 1987, Vol. 17, pp.34-56, https://doi.org/10.1016/S0166-2481(08)70079-5

L.T. Shirshova, D.A. Gilichinsky , N.V. Ostroumova, & Yermolayev, A. M. (2015). Application of spectrophotometry to quantification of humic substances in permafrost sediments.Kriosfera Zemli. 2015, vol. XIX, No. 4, pp. 96–102

M.A. Mohamed, J. J., A.F. Ismail, M.H.D. Othman, M.A. Rahman. (2017). Fourier Transform Infrared (FTIR) Spectroscopy. Membrane Characterization.2017, pp. 3-29, doi: 10.1016/B978-0-444-63776-5.00001-2

McCarthy, M. D., & Bronk, D. A. (2008). Analytical Methods for the Study of Nitrogen (2nd ed.).

Megan Fenton, C. A., Quirine Ketterings. (2008). Soil Organic Matter. Agronomy Fact Sheet Series, 41, http://nmsp.css.cornell.edu

Orsetti, S., Quiroga Mde, L., & Andrade, E. M. (2006). Binding of Pb(II) in the system humic acid/goethite at acidic pH. *Chemosphere, 65*(11), 2313-2321. doi: 10.1016/j.chemosphere.2006.05.009

Pettit, R. E. (2012). Organic matter, humus, humate, humic acid, fulvic acid and humin: their importance in soil fertility and plant health. Retrieved from https://humates.com/pdf/ORGANICMATTERPettit.pdf

Pirbadian, S., Barchinger, S. E., Leung, K. M., Byun, H. S., Jangir, Y., Bouhenni, R. A., . . . El-Naggar, M. Y. (2014). Shewanella oneidensis MR-1 nanowires are outer membrane and periplasmic extensions of the extracellular electron transport components. *Proc Natl Acad Sci U S A, 111*(35), 12883-12888. doi: 10.1073/pnas.1410551111

Purmalis, O., & Klavins, M. (2013). Comparative study of peathumic acidsby using uv spectroscopy. European Scientific Journal. 2013, special edition No.3, ISSN: 1857 - 7431, doi: http://dx.doi.org/10.19044/esj.2013.v9n21p%25p

Riemer, J., Hoepken, H. H., Czerwinska, H., Robinson, S. R., & Dringen, R. (2004). Colorimetric ferrozine-based assay for the quantitation of iron in cultured cells. *Anal Biochem, 331*(2), 370-375. doi: 10.1016/j.ab.2004.03.049

Siddharth Natarajan. (2016). Organic Agro Inputs and Their benefits for soil and Plant Health. Retrieved from https://www.slideshare.net/SiddharthN atarajan1/winatu als-presentation.

Shi, L., Dong, H., Reguera, G., Beyenal, H., Lu, A., Liu, J., . . . Fredrickson, J. K. (2016). Extracellular electron transfer mechanisms between microorganisms and minerals. *Nat Rev Microbiol, 14*(10), 651-662. doi: 10.1038/nrmicro.2016.93

T I Tong, N. L. L. F., S Mohd, A Sulaeman (2015). Characterization of Soil Organic Matter in Peat Soil with Different Humification Levels using FTIR. IOP Conference Series: Materials Science and Engineering 136 (2016) 012010, doi: 10.1088/1757-899X/136/1/012010

Tsutsuki, K., & Kuwatsuka, S. (1979). Chemical studies on soil humic acids. *Soil Science and Plant Nutrition, 25*(4), 501-512. doi: 10.1080/00380 768.19 79.10433192

Ummo Fooken , G. L. (2003). An IR Study of Humic Acids Isolated from Sediments and Soils. Marine Biodiversity. 2003, ISSN: 1867-1624, Vol. 32, Issue 1–2, pp 183–189, doi: https://doi.org/10.1007/BF03043094

Wang, S, Mulligan, C. N.(2006). Effect of natural organic matter on arsenic release from soils and sediments into groundwater. Environ Geochem Health 28(3), 197-214. 10.1007/s10653-005-9032-y.

Wee, S. K. (2014). Novel Pathway for Microbial Fe(III) Reduction: Electron Shuttling through Naturally Occurring Thiols. Georgia Institute of Technology 150(17).

Xu, W., Hu, Pei, Zhou, Shun-gui, Li, Xiaomin, Li, Yan-hong. (2009). Electron shuttling function of dissolved organic matter. Environmental Science. 2009, 30(8):2297-301, https:// www. ncbi. nlm.nih.gov/pubmed/19799291

APPENDIX

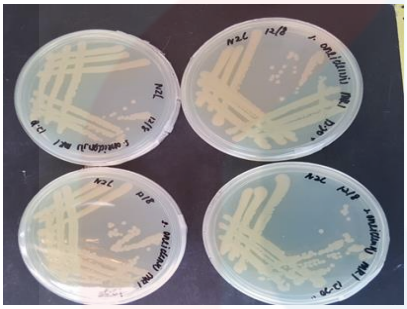


Figure A.1: Subculture of *S.oneidensis* MR-1

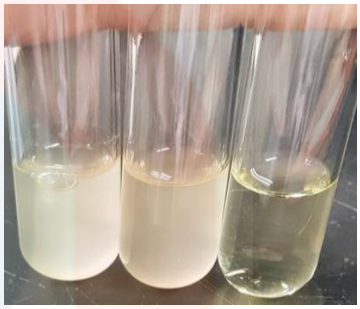


Figure A.2: Seed culture of *S.oneidensis* MR-1

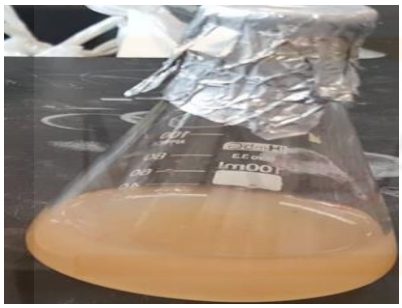


Figure A.3: Seed culture of *S.oneidensis* MR-1 (50 mL)

Figure A.4: Qualify Fe(II) concentration by Ferrozine assay

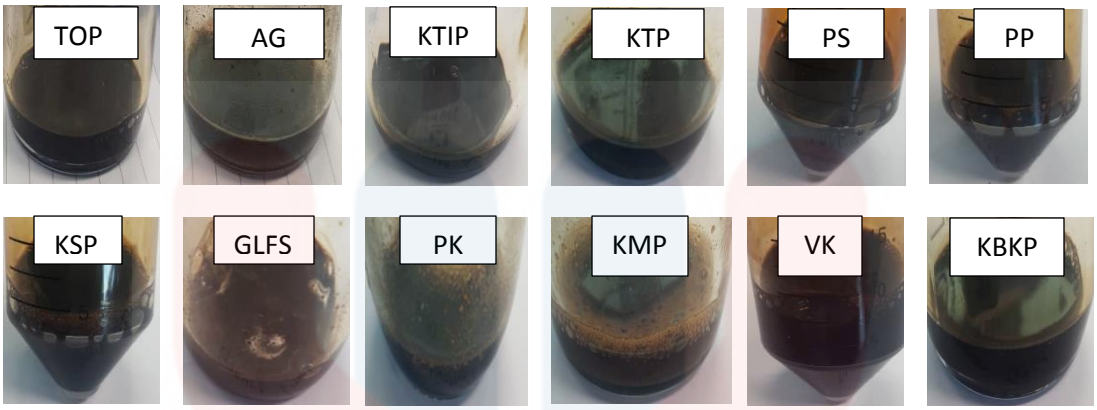


Figure A.5: Extracted humic acids solution from various soil source

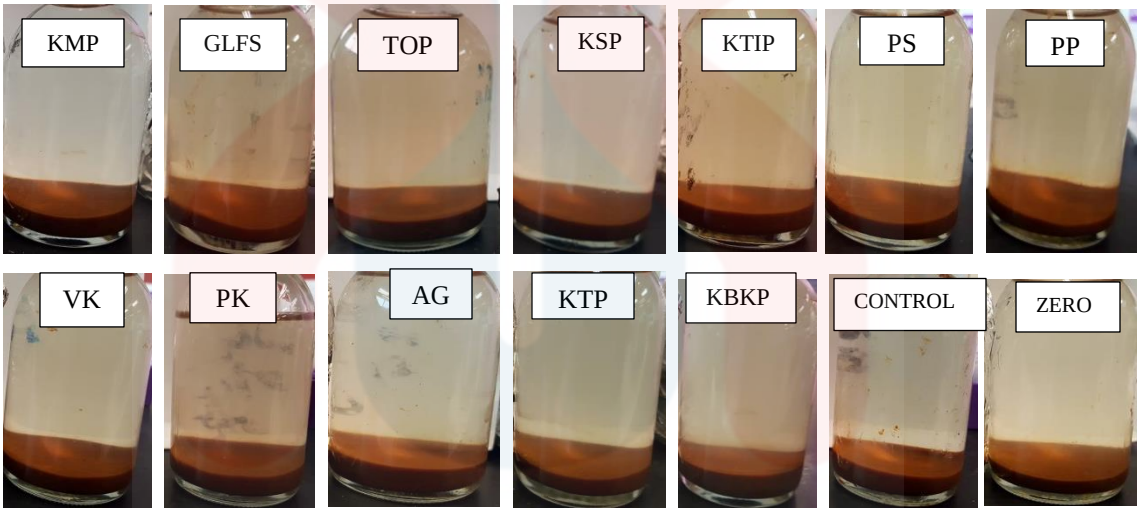


Figure A.6: 12 various humic acids on microbial Fe(III) reduction with control and zero

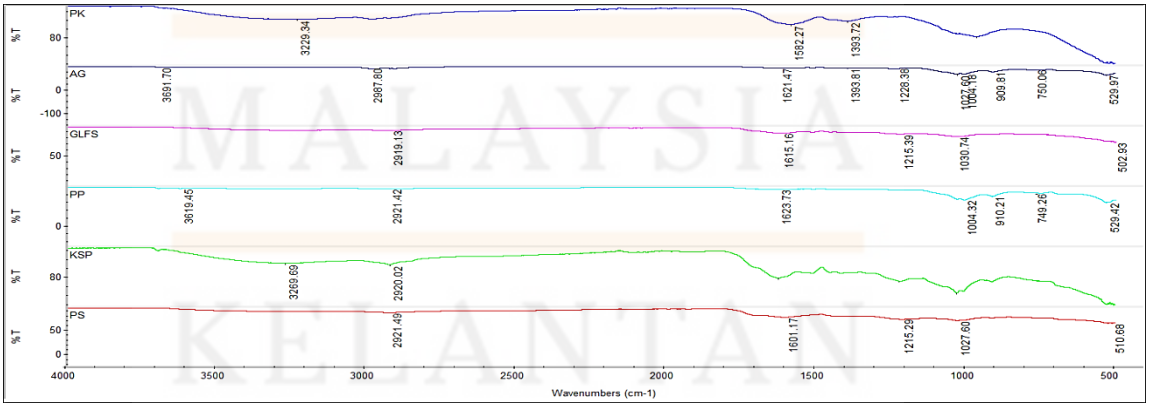


Figure A.7: IR spectral of extracted humic acids (PS, GLFS, KSP, AG, PP and PK)