ISOLATION AND CHARACTERIZATION OF IRON REDUCING BACTERIA FROM ANTARTICA AND GENTING HIGHLAND

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DECLARATION

I declare that this thesis entitled "Isolation and characterization of iron reducing bacteria from Antarctica and Genting highland" is the result of my own research except as cited in the references.

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Pengasingan dan pencirian bakteria pengurangan besi dari Antartika dan Genting highland

ABSTRAK

Kajian ini menyiasat kesan aktiviti pengurangan zat besi dalam komuniti mikrob dari Antartika dan Genting Highland, menangani persoalan kepelbagaian mikroorganisma extremophilic dan penyesuaian fungsi dalam persekitaran ini. Hipotesis menunjukkan keupayaan pengurangan besi yang berbeza sejajar dengan niche ekologi tertentu. Didorong oleh pemahaman yang lebih luas tentang dinamik ekosistem, kajian ini menggunakan ujian pengurangan besi, pengasingan berasaskan pencairan bersiri, pewarnaan gram, dan ujian biokimia. Keputusan menunjukkan potensi pengurangan besi dinamik dalam kedua-dua persekitaran, dengan Antartika 1 mempamerkan mikroorganisma positif Gram dan Antartika 2 dan Genting Highland memaparkan mikrob gram-negatif. Ujian oksidase positif di semua sampel menunjukkan kehadiran cytochrome c oxidase, menonjolkan keupayaan pernafasan aerobik. Ujian catalase menunjukkan perbezaan, dengan Genting Highland positif dan Antartika negatif, mencabar hipotesis awal dan mendorong penilaian semula strategi metabolik mikrob. Penemuan ini menyumbang kepada pemahaman yang lebih mendalam tentang ekologi mikrob extremophilic, menekankan kepentingan kepelbagaian mikrob dalam ekosistem yang unik. Hasil ujian catalase yang tidak dijangka menekankan keperluan untuk penerokaan lebih lanjut ke dalam kepelbagaian metabolik mikrob dan menyerlahkan kepentingan mempertimbangkan pelbagai faktor dalam kajian komuniti mikrob. Penyelidikan ini mempunyai implikasi yang lebih luas untuk pengurusan alam sekitar dan aplikasi bioteknologi, kerana ia memperkenalkan interaksi rumit penyesuaian mikrob dalam persekitaran yang melampau, menggesa pendekatan yang lebih nuansa untuk analisis komuniti mikrob.

Kata kunci: Ekstremopiles, pengurangan besi, komuniti mikrob, niche ekologi, kepelbagaian metabolik.

Isolation and characterization of iron reducing bacteria from Antarctica and Genting highland

ABSTRACT

This research focuses on isolating and characterizing iron-reducing bacteria from sediment samples collected in Antarctica and Genting Highland using an enrichment strategy addressing the question of extremophilic microorganism diversity and functional adaptations in these environments. The hypothesis suggests distinct iron reduction capabilities aligned with specific ecological niches. Motivated by a broader understanding of ecosystem dynamics, the study employs iron reduction assays, serial dilution-based isolation, gram staining, and biochemical testing. Results reveal dynamic iron reduction potential in both environments, with Antarctica 1 exhibiting Gram-positive microorganisms and Antarctica 2 and Genting Highland displaying Gram-negative microbes. The positive oxidase test across all samples indicates the presence of cytochrome c oxidase, highlighting aerobic respiration capabilities. The catalase test shows differences, with Genting Highland positive and Antarctica negative, challenging initial hypotheses and prompting a reevaluation of microbial metabolic strategies. These findings contribute to a deeper understanding of extremophilic microbial ecology, emphasizing the significance of microbial diversity in unique ecosystems. The unexpected catalase test outcomes underscore the need for further exploration into microbial metabolic diversity and highlight the importance of considering multiple factors in microbial community studies. This research has broader implications for environmental management and biotechnological applications, as it unveils the intricate interplay of microbial adaptations in extreme environments, urging a more nuanced approach to microbial community analysis.

Keywords: Extremophiles, iron reduction, microbial communities, ecological niches, metabolic diversity.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST ABBREVIATIONS

DIRB	Dissimilatory Iron Reducing Bacteria
16S rRNA	16S ribosomal ribonucleic acid
NaCL	Sodium Chloride
LB broth	Luria Bertani broth
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Di-potassium hydrogen phosphate
$CoSO_4\ 7H_2O$	Cobalt(ii) sulfate heptahydrate
$NiCl_2\ 6H_2O$	Nickel (II) chloride hexahydrate
H_3BO_3	Boric acid
$ZnSO_4.7H_2O$	Zinc sulfate heptahydrate
$Na_2MoO_4\text{-}2H_2O$	Sodium molybdate dihydrate
$CuSO_4\text{-}5H_2O$	Copper (II) sulfate pentahydrate
$MnSO_4\ H_2O$	Manganese (II) sulfate monohydrate
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
$CaCl_2\text{-}2H_2O$	Calcium chloride dehydrate
$FeSO_4.7H_2O$	Iron (II) sulfate heptahydrate
$NaSeO_4$	Sodium selenite
$NaHCO_3$	Sodium bicarbonate
$[(NH_4)2SO_4]$	Ammonium sulfate
N	Normality
NaOH	Sodium hydroxide
HCl	Hydrochloric acid

HFO	Hydrous ferric oxide
H_2O_2	hydrogen peroxide
FeCl3·6H2O	Chloride hydrate
H_2O	Water
GH	Genting Highland
ATT	Antarctica
P1	Point 1
P3	Point 3
dH2O	Distilled water
O_2	Oxygen
IRB	Iron reducing bacteria

LIST OF SYMBOLS

℃	Degree Celsius
%	Percentage
℉	Fahrenheit
µl	microliter
g/L	gram per liter
mM	millimolar
mL	milliliter
L	Liter
g	Gram

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Antarctica stands out as the coldest, driest, windiest, and iciest of all known habitats, ranking among the most extreme environments globally. Within this inhospitable terrain, psychrophilic bacteria emerge as the predominant microbial group, contributing significantly to nutrient recycling. A decade later, research revealed that heterotrophic bacteria not only endure but actively reproduce in Antarctica's low temperatures, ranging from 1 to 2 degrees Celsius (Miteva, 2008). Building on these foundational studies, the application of a polyphasic taxonomic approach led to the identification of novel bacterial species on the Antarctic continent. Subsequently, the introduction of metagenomic techniques uncovered a greater diversity of microbes across various Antarctic habitats. Notably, the NCBI database houses 4964 16S rRNA gene sequences of bacteria from Antarctica. However, it is noteworthy that only 498 of these sequences correspond to cultivable isolates obtained from the Antarctic region (Kumar Chattopadhyay, Sathyanarayana Reddy, & Shivaji, 2014).

Iron (Fe), a predominant element in Earth's crust, undergoes cycling primarily between its reduced ferrous iron (II) and oxidized ferric iron (III) forms through the activities of virtually all living organisms (Melton, Swanner, Behrens, Schmidt, & Kappler, 2014). The capability of microorganisms to facilitate the dissimilatory reduction of ferric iron (III) has played a pivotal role in the evolutionary processes shaping the Earth (Obuekwe, Westlake, Cook, & William Costerton, 1981). Microbes exhibiting such capabilities, known as dissimilatory iron-reducing bacteria (DIRB), are of significant interest due to their involvement in environmentally crucial processes, including the biogeochemical cycling of iron and carbon, the bioremediation of organohalides and radionuclides, and the generation of electricity in microbial fuel cells (D. R. Lovley, Holmes, & Nevin, 2004). The microbial reduction of iron (II) is considered one of the earliest respiratory

processes to have evolved on Earth, reflecting the deep-seated presence of iron (III)-reducing microorganisms across the prokaryotic domain (Lonergan et al., 1996).

In the context of microbial metal respiration, the fascinating relationship with psychrophilic bacteria adds another layer to the intricate web of microbial interactions in extreme environments like Antarctica. The adaptability of psychrophilic bacteria to the continent's cold conditions aligns with their role in metal respiration, potentially influencing the redox state of metals in polar environments. While much of the research on dissimilatory iron-reducing bacteria (DIRB) has focused on mesophilic and thermophilic organisms, understanding how psychrophilic bacteria contribute to microbial metal respiration becomes imperative in the context of polar ecosystems. These cold-adapted microorganisms may play a crucial role in influencing metal mobility and biogeochemical cycles in cold environments, where traditional expectations about microbial activity might not directly apply. Investigating the interactions between psychrophilic bacteria and metal respiration enhances our understanding of microbial adaptation to extreme cold, offering insights into their potential contributions to essential environmental processes in polar regions.

1.2 Problem Statement

The extreme environments of Antarctica and Genting Highland present unique challenges and opportunities for microbial life. While iron-reducing bacteria have been identified in various habitats globally, there is a gap in our understanding of these microorganisms specifically in polar and high-altitude regions. Investigating the iron reduction activities in bacterial cultures from Antarctica and Genting Highland is crucial for filling this knowledge gap and comprehending the adaptability of microorganisms to extreme climates.

1.3 Objective

The objectives of the research are:

1. To isolate iron reducing bacteria from Antarctica and Genting Highland sediment samples through enrichment strategy.
2. To characterize iron reducing bacteria isolated from Antarctica and Genting Highland sediment samples.

1.4 Scope of Study

This research focuses on the isolation and characterization of iron-reducing bacteria from two contrasting environments: the cold, pristine expanses of Antarctica and the high-altitude conditions of Genting Highland. The scope encompasses the evaluation of iron reduction activities in bacterial cultures from these regions using the ferrozine assay. Furthermore, the study involves the isolation and detailed characterization of individual bacterial colonies through gram staining and biochemical tests. The scope extends to the exploration of microbial diversity and functional attributes specifically related to iron reduction in these unique environments.

1.5 Significance of study

Understanding the presence and activity of iron-reducing bacteria in Antarctica and Genting Highland holds significant ecological and biotechnological implications. The findings contribute to our knowledge of microbial adaptation to extreme climates and advance our understanding of biogeochemical cycling in polar and high-altitude regions. Moreover, the study has potential applications in environmental management, bioremediation strategies, and the broader field of microbiology. The significance lies not only in expanding our scientific knowledge but also in identifying potential tools for addressing environmental challenges.

CHAPTER 2

LITERATURE REVIEW

2.1 Psychrophilic bacteria

Psychrophiles are bacteria that thrive in cold environments, typically below 20 °C. Coined by Schmidt-Nielsen in 1902, the term "psychrophile" means "cold-loving" (from the Greek words psychros, meaning cold, and philes, meaning loving) (Ingraham & Stokes, 1959). However, it is essential to note that despite this label, most psychrophiles tend to grow above 20 °C, but not surpassing 30 °C, making them more accurately described as cold-tolerant rather than exclusively "cold-loving." Previously, cold-tolerant bacteria were categorized as either psychrotrophs, capable of growing between 0 to 30 °C, or psychrophiles, thriving between 1 to 20 °C with an optimum around 16 °C. Nevertheless, the current classification disregards this distinction, collectively terming all cold-tolerant bacteria as psychrophiles. Recent studies on bacteria from diverse Antarctic biotopes challenge traditional classifications, indicating that a significant percentage (40-70%) can grow in the temperature range of 1-30 °C, leading to their characterization as psychrotolerant organisms (Romanovaskaia, Tashirev, Gladka, & Tashireva, 2012). This evolving understanding emphasizes the complexity of bacterial adaptation to cold environments, particularly in extreme habitats like Antarctica.

2.2 Mechanism of bacterial adaptation to low temperature

Cold-tolerant bacteria employ sophisticated strategies to maintain membrane fluidity and ensure cellular functionality in low-temperature environments. One crucial adaptation involves adjusting the composition of the cell membrane's lipid bilayer, specifically manipulating the ratio of saturated to unsaturated fatty acids. Saturated fatty acids, with straight hydrocarbon chains, tend to pack tightly, making the membrane rigid. In contrast, unsaturated fatty acids, characterized by kinks in their chains due to double bonds, introduce flexibility, preventing close packing and enhancing membrane fluidity (Chattopadhyay, 2006). By incorporating a higher proportion of unsaturated fatty acids in response to cold temperatures, these bacteria effectively counteract the tendency of the lipid bilayer to become too rigid. This dynamic adjustment allows the membrane to maintain a semi-fluid state, facilitating essential cellular processes and preserving membrane integrity. The role of membrane fluidity in cold-tolerant bacteria extends beyond physical properties to impact overall cellular functionality. A flexible membrane is vital for maintaining proper permeability, ensuring the efficient transport of nutrients and waste products. Additionally, membrane-associated proteins, integral for various cellular functions, rely on an optimal degree of fluidity. Cold-tolerant bacteria have evolved temperature-sensitive regulatory mechanisms to dynamically modulate membrane composition. Enzymes like desaturases, involved in synthesizing unsaturated fatty acids, exhibit increased activity in response to lower temperatures. This regulatory flexibility allows these bacteria to fine-tune their membrane structure, demonstrating a remarkable ability to adapt and thrive in cold environments.

2.3 Dissimilatory Iron reduction

Dissimilatory iron reduction is the utilize of iron (III) as a terminal electron acceptor to produce energy when respire anaerobically. Dissimilatory iron reduction is mostly located in the subsurface environments such as soils and sediments where a couple of electron acceptors are available for microorganisms besides iron. Previous research reports that the free energy generated by reducing different electron acceptors may determine the sequence by which microorganisms prefer to select in the subsurface environment (Reeburgh, 1983). The capacity for the reduction of Fe (III) has evidently evolved through multiple instances, with phylogenetically distinct Fe (III) reducers employing diverse methods for this reduction process, as supported by both microbiological

and geochemical evidence. The ability of microorganisms to reduce Fe (III) has been recognized since the early twentieth century. However, it was not until the 1980s that scientists uncovered the phenomenon of certain microorganisms harnessing energy through the oxidation of hydrogen or organic chemicals to sustain their growth. Methanogenesis stands out as one of the primary recognized modes of respiration in soils and sediments, encompassing processes such as oxygen reduction, nitrate reduction, and sulphate reduction (Vargas, Kashefi, Blunt-Harris, & Lovley, 1998)

2.4 Microbial Iron Reduction with Hydrous Ferric Oxide

2.4.1 Iron (Hydr)oxides in Anaerobic Environments: Diversity and Mechanisms of Bacterial Fe (III) Respiration

Iron (hydr)oxides are widespread in the environment, with concentrations ranging from one to several hundred grams per kilogram in aerobic soils (Cornell & Schwertmann, 1996). In anaerobic, nonsulfidogenic conditions, the primary method of reducing iron (hydr)oxides is through bacterial Fe (III) respiration (D R Lovley, 1991). Dissimilatory iron-reducing bacteria link the oxidation of H2 or organic carbon to the reduction of Fe (III), generating energy in the form of adenosine triphosphate (ATP) (Lovley and Phillips 1988, Lovley 1991, Nealson and Saffarini 1994).

2.4.2 Iron Reduction Impact: Biogeochemical Consequences for Metal Cycles and Contaminant Fate in Subsurface Environments

The formation and transformations of iron (hydr)oxides play a crucial role in determining the fate of nutrients and metals in the environment. Dissimilatory iron-reducing bacteria can either enhance (release of sorbed ions) or diminish (reductive immobilization via redox active metabolites) contaminant fate and transport in subsurface environments. The strong reducing capacity of ferrous Fe shapes the fate of Fe(II) following dissimilatory iron reduction, impacting metal cycles. The ultimate destiny of Fe(II) significantly influences the biogeochemical cycling of iron, nutrients, and contaminants. The pathways of secondary mineralization for Fe(II) after dissimilatory iron reduction are controlled by the supply rate and concentration of Fe(II) in solution. Variations in microbial bioavailability among different iron (hydr)oxides can substantially impact the fate of Fe (II) and its subsequent secondary mineralization.

2.5 Antarctica and Genting highland

2.5.1 Environmental conditions in Antarctica

Antarctica, known for its extreme climate, is the coldest, driest, windiest, and iciest continent on earth (Benninghoff, 1987). The temperature can plummet to as low as -80°C (-112°F), and the continent is covered by a thick ice sheet. The harsh conditions, coupled with limited precipitation, create a unique and challenging environment. Microbial life in Antarctica is predominantly represented by psychrophilic bacteria, which thrive in cold temperatures.

Prior studies in Antarctica have revealed the prevalence of psychrophilic bacteria, including those with iron-reducing capabilities. These microorganisms are crucial for nutrient cycling in this extreme environment. Research conducted on microbial communities in Antarctic soils, sediments, and ice has highlighted the presence of iron-reducing bacteria adapting to the cold conditions. These studies often employ molecular techniques, such as 16S rRNA gene sequencing, to identify and characterize the diverse microbial communities.

In Antarctica, extensive research endeavors have concentrated on the isolation and characterization of psychrophilic bacteria, particularly those exhibiting iron-reducing capabilities (Koh et al., 2017). Employing metagenomic methodologies, studies have delved into the intricate genetic diversity prevalent in microbial communities residing in Antarctic soils and sediments (Tveit, Urich, & Svenning, 2014). Notably, specific iron-reducing bacteria, exemplified by members from the Shewanella and Geobacter genera, have been meticulously identified and documented as integral components of the region's microbial landscape (Lauro et al., 2011).

2.5.2 Environmental conditions in Genting Highland

Genting Highlands, in contrast, represents a tropical montane region located in Malaysia. Situated at a higher altitude, it experiences a cooler climate compared to the surrounding lowlands. The temperature in Genting Highlands ranges from 16°C

to 25°C (61°F to 77°F), making it significantly milder than Antarctica. The region is characterized by lush forests, providing a diverse habitat for various microorganisms.

While Genting Highlands may not exhibit extreme cold conditions like Antarctica, the relatively cooler climate, coupled with unique ecological niches, creates an environment conducive to studying microbial communities. Understanding the microbial diversity in this montane region, especially those involved in iron reduction, can contribute to broader knowledge about the adaptability of microorganisms to varying environmental conditions.

2.6 Microbial isolation and characterization

Microbial isolation and characterization, especially of iron-reducing bacteria, play a crucial role in understanding their ecological functions and potential applications. Numerous studies have employed diverse methodologies to isolate and identify these bacteria from various environments, ranging from soils to extreme habitats like Antarctica. Isolation techniques often involve enrichment cultures, where specific growth conditions favor the proliferation of iron-reducing bacteria. Subsequent characterization is typically achieved through a combination of morphological assessments, Gram staining, and biochemical tests (Emerson & Moyer, 1997). Gram staining, a fundamental microbiological technique, classifies bacteria based on the characteristics of their cell walls, distinguishing them as either Gram-positive or Gram-negative. Biochemical tests, such as oxidase and catalase assays, further refine bacterial identification by examining specific metabolic traits. The combined use of these techniques facilitates the accurate isolation and characterization of iron-reducing bacteria, providing insights into their diversity, physiology, and potential environmental roles.

2.7 Ferrozine assay

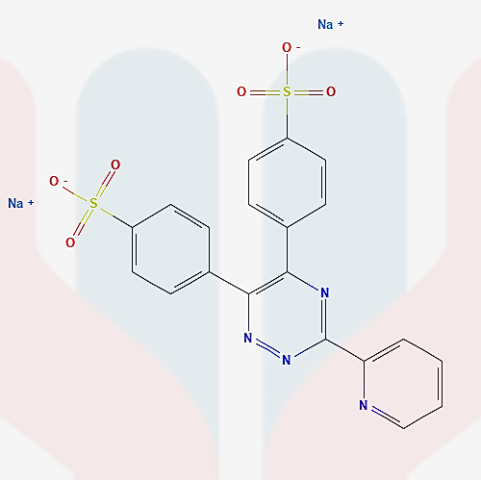


Figure 1: Chemical structure of Ferrozine
(NCBI, 2024)

The Ferrozine assay is a widely used method for determining ferrous (Fe^2+) and total iron concentrations in geomicrobiological studies due to its efficiency and reliability. Ferrozine, a bidentate iron chelator, forms a stable and specific magenta complex with iron ions, particularly ferrous iron. This complex absorbs light at 562 nm, allowing for the quantification of iron concentrations using a spectrophotometer.

The principle behind the Ferrozine assay involves the reaction between ferrous iron in the sample and ferrozine. When ferrozine binds to ferrous iron, it forms a distinct magenta-colored complex. The intensity of this color is directly proportional to the concentration of ferrous iron in the sample. Therefore, by measuring the absorbance of the complex at 562 nm, researchers can precisely determine the amount of ferrous iron present.

One of the key advantages of the Ferrozine assay is its sensitivity, making it particularly suitable for detecting low concentrations of ferrous iron in various environmental samples. The colorimetric approach simplifies the quantification process, and the assay is known for its accuracy and reproducibility. The method has been applied in studies exploring microbial iron reduction, biogeochemical cycling, and environmental monitoring.

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

Soil sample from Antarctica and Genting Highland, Luria Bertani agar (16 g/L agar, 10 g/L sodium chloride (NaCL), 5 g/L yeast extract), LB broth (10 g/L tryptone, 10 g/L sodium chloride (NaCL), 5 g/L yeast extract), distilled water, M1 medium, phosphate buffer, potassium dihydrogen phosphate (KH_2PO_4), di-potassium hydrogen phosphate (K_2HPO_4), metal supplement, cobalt(ii) sulfate heptahydrate ($CoSO_4$ $7H_2O$), nickel(II) chloride hexahydrate ($NiCl_2$ $6H_2O$), natrium chloride (NaCl), trace element solution boric acid (H3BO3), zinc sulfate heptahydrate (ZnSO4.7H2O), sodium molybdate dihydrate (Na_2MoO4-$2H_2O$), copper (II) sulfate pentahydrate ($CuSO4$-$5H_2O$), manganese (II) sulfate monohydrate (MnSO4 H2O), basal salts, magnesium sulfate heptahydrate (MgSO4.7H2O), calcium chloride dehydrate ($CaCl_2$-$2H_2O$), EDTA disodium salt, iron (II) sulfate heptahydrate (FeSO4.7H2O), mixed amino acids, L-serine, L-arginine, L-glutamic acid, 30%, 115mM sodium selenite (NaSeO4), 200mM sodium bicarbonate (NaHCO3), ammonium sulfate [(NH4)2SO4], yeast extracts, 10 N sodium hydroxide (NaOH), 5 N hydrochloric acid (HCl), hydrous ferric oxide (HFO), ferrozine assay, ethanol, 30% glycerol, crystal violet, iodine solution, acetone, safranin, hydrogen peroxide (H_2O_2), N,N,N',N'-tetramethyl phenylenediamine dihydrochloride.

3.2 Apparatus

Eppendorf tubes, falcon tubes, marker, parafilm, sterile microcentrifuge tubes, sterile inoculating loop, syringe, needles, micropipette tips, aluminum foil, serum bottles, decapper, Schott bottles, beakers, measuring cylinders, droppers, spatula, test tubes, test tube rack, mask, gloves, cuvettes, Bunsen burner, microcentrifuge tube rack, conical flasks, petri dishes, filter paper, and microscope slides.

3.3 Methods

3.3.1 Preparation of M1 minimal medium

To prepare M1 minimal medium, phosphate buffer, metal supplement, trace element solution, basal salts, mixed amino acids, yeast extract, 115 mM NaSeO4, and 200 mM NaHCO3 were used.

For the preparation of 1.0 L phosphate buffer, 30.0g potassium dihydrogen phosphate (KH2PO4), 66.1g di-potassium hydrogen phosphate (K2HPO4), and 800 mL of distilled water were added. The phosphate buffer was adjusted to pH 7 with the addition of NaOH and HCl as needed. It was brought to a final volume of 1.0 L by the addition of dH2O. This solution was stored at 4°C.

To prepare the metal supplement, 1.41g cobalt (II) sulfate heptahydrate (CoSO4.7H2O), 1.98g nickel (II) chloride hexahydrate (NiCl2.6H2O), 0.58g sodium chloride (NaCl), and 100 mL of dH2O were added. The solution was autoclaved and stored at 4°C.

For the preparation of the trace element solution, 2.8g boric acid (H3BO3), 0.24g zinc sulfate heptahydrate (ZnSO4.7H20), 0.75g sodium molybdate dihydrate (Na2MoO4.2H20), 0.042g copper (II) sulfate pentahydrate (CuSO4.5H2O), and 0.17g manganese (II) sulfate monohydrate (MnSO4.H2O) were mixed with 1.0 L distilled water to avoid the occurrence of precipitation. The metal supplement was filtered sterile by a 0.22 um nano filter and stored at 4°C.

To prepare the basal salts solution, 10 mL of the previously prepared trace element solution, 2.0g magnesium sulfate heptahydrate (MgSO4.7H2O), 0.57g calcium chloride dehydrate (CaCl2.2H2O), 0.20g EDTA disodium salt, and 0.012g iron (II) sulfate heptahydrate (FeSO4.7H20) were mixed with 800 mL of dH2O. It was stored at 4°C.

0.2g each of L-serine, L-arginine, and L-glutamic acid were added to 100 mL of dH2O for the preparation of the mixed amino acids solution. This solution was autoclaved at 121°C and stored at 4°C. To prepare 115mM sodium selenite (NaSeO4), 1.086g NaSeO4 was added to 500 mL of distilled water. 4.2g sodium bicarbonate (NaHCO3) was added to 250 mL of dH2O to prepare 200mM NaHCO3.

To prepare 500 mL of M1 medium, 0.95g of ammonium sulfate was added to a media bottle, followed by 7.5 mL phosphate buffer, 50 mL basal salts, and 438 mL of distilled water. 0.05 mL each of 115mM NaSeO4 solution and metal supplement, and 2.5g of yeast extract were added to the

bottle. The pH of the solution was adjusted to pH 7 with 10 N NaOH. M1 medium was autoclaved at 121°C, and this medium can be stored at this stage for months before use. Prior to inoculation, 5 mL each of the mixed amino acid solution and 200mM NaHCO3 were added. Electron acceptors, 50 mL of Hydrous Ferric Oxide were added.

3.3.2 Preparation of Hydrous Ferric Oxide

54 grams of iron (III) chloride hydrate (FeCl3·6H2O) were dissolved in 500 mL of sterile H2O to prepare a Hydrous Ferric Oxide mixture (HFO). The precipitation of Fe (III) oxides occurred by increasing the pH to 7.0 through the addition of 10 N NaOH. The resulting 0.4 M HFO mixture was then stored in the dark until needed.

3.3.3 Bacterial growth conditions (Enrichment 1)

0.1g of soil from Antarctica and Genting Highland were each diluted in 2 ml of sterile distilled water. Next, 100 µl of each soil sample was transferred into serum bottles containing 50 ml of M1 minimal medium supplemented with yeast extract as the electron donor and Hydrous Ferric Oxide and iron (III) citrate as the electron acceptor.

In total, eight serum bottles were prepared, including duplicate anaerobic controls, duplicate samples from ATT, and duplicate samples from GH (P1) as well as GH (P3). All bottles were tightly sealed, wrapped with aluminum foil, and stored at 4°C for further growth.

3.3.4 Quantification of iron (III) reduction activities via ferrozine assay

The control and bacterial cultures from the eight serum bottles were tested for iron (III) reduction activity. They were grown anaerobically at 4°C on M1 medium supplemented with yeast extract as the electron donor and Hydrous Ferric Oxide and iron (III) citrate as the electron acceptor.

For the assay, 200 µL of samples were placed into microcentrifuge tubes with 1 mL of 0.5 N HCl and left in the dark for 15 minutes. Subsequently, 50 µL of the sample was added to 950 µL of Ferrozine in new microcentrifuge tubes. Fe (III) reduction was monitored for total Fe (II) production

over time using the Ferrozine technique (Stookey, 1970) after extraction with HCl (Lovley & Phillips, 1986).

Absorbance readings were recorded and measured using a spectrophotometer at 562 nm, and the adjusted blank was set to zero using dH2O. The concentration of produced iron (II) can be calculated by subtracting the final optical density reading of the sample with 0.016 and then dividing by 0.1999 (Wee, 2014).

$$\text{Iron (II) concentration} = \frac{(\boldsymbol{final\ absorbance\ reading - 0.016})}{\mathbf{0.1999}}$$

3.3.5 Enrichment 2

The procedures for the second enrichment are the same as those in Enrichment 1, except that for the samples, 100 µl of sample was taken from each serum bottle from Enrichment 1. These samples were then transferred into new serum bottles containing 50 ml of M1 minimal medium, supplemented with yeast extract as the electron donor, and Hydrous Ferric Oxide as the electron acceptor. A total of eight serum bottles were prepared, following the same duplication scheme as in Enrichment 1. This included duplicate anaerobic controls, duplicate samples from ATT, and duplicate samples from GH (L1) and GH (L3).

3.3.6 Isolation of pure culture

3.3.6.1 Serial dilution and spread plate

Serial dilution was performed to achieve distinct and countable single colonies. Six test tubes were prepared with 9 mL of LB broth, resulting in dilutions ranging from 10^{-1} to 10^{-6} for each sample. In sterile test tubes, 9 mL of LB broth was added. Subsequently, 1 mL of bacterial culture from the incubation media was transferred to initiate the dilution process.

From the dilutions (10^{-2}, 10^{-4}, and 10^{-6}), 100 µL of the cell suspension was selected and spread onto LB agar, including an experimental control. The spread plates were incubated aerobically at 4°C for a duration of 3 days. Bacterial growth was monitored by observing the appearance of individual colonies after the incubation period.

3.3.7 Characterization of iron reducing bacteria

3.3.7.1 Gram staining

One loopful of saline water was placed on a dry, clean glass slide. Using a sterile loop, one colony of bacteria from ATT 1&2 and GH L1(1) & L3(2) was taken from agar plates and smeared into the saline water on the slide. The smears were allowed to air-dry.

Subsequently, the smears were stained with crystal violet and left for 1 minute. Afterward, they were rinsed with water. The smears were then stained with iodine for 1 minute and again rinsed with water. Following this, a careful rinse with acetone for 30 seconds was performed, followed by another rinse with water.

Next, the smears were counter-stained with safranine for 1 minute, rinsed with water, and the slide was dried. Finally, the prepared slide was observed under the microscope using 10X, 40X, and 100X power lenses.

3.3.7.2 Biochemical test

1. **Catalase test**

A small amount of growth from the culture was placed onto a clean microscope slide. Care was taken while using colonies from the agar plate to avoid scraping up any contaminating agar, as this could give a false positive. A few drops of H2O2 were added to the smear, and the mixture was stirred with a toothpick.

A positive result was indicated by the rapid evolution of O2, evidenced by bubbling. A negative result was observed when there were no bubbles or only a few scattered bubbles. The slide was disposed of in the biohazard glass disposal container, and any used toothpicks were disposed of in the Pipet Keeper.

2. Oxidase test

With a sterile filter paper, a small colony was obtained from an agar slant or plate. One drop of reagent was placed onto the culture on the swab. Positive reactions turned the bacteria violet to purple immediately or within 10 to 30 seconds. Delayed reactions were ignored.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Quantification of iron (III) reduction activities via ferrozine assay

4.1.1 Antarctica

Figure 4.1 shows the 1st enrichment of iron (III) reduction of bacteria from soil sample from Antarctica 1 and 2 with abiotic control using yeast extract as sole electron donor and Hydrous Ferric Oxide (HFO) as the sole electron acceptor. Iron (III) reductions were indicated by the production of iron (II). Error bars represented standard deviations.

Contrastingly, in Antarctica 1 and 2, where live bacteria are present, a more stable pattern is evident. Antarctica 1 maintains consistent iron reduction values of 0.210mM, 0.300mM, and 0.240mM on Days 0, 4, and 7, respectively. Similarly, Antarctica 2 exhibits stability with values of 0.265mM, 0.285mM, and 0.220mM on the same days. The minor decrease in Antarctica 2 on Day 7 may be attributed to microbial adaptation or other environmental influences. Extreme environmental factors such as temperature, pH, and substrate availability can significantly impact microbial activities.

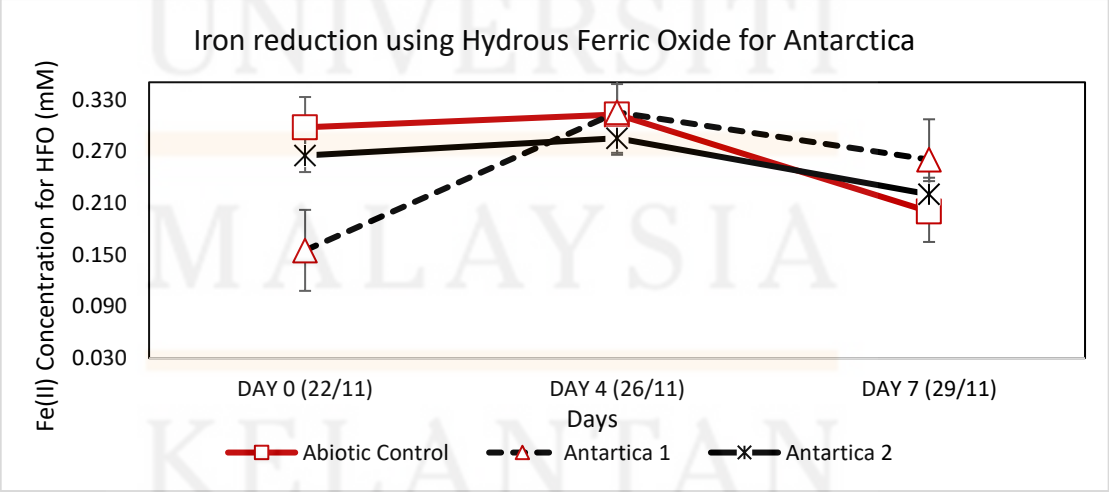


Figure 4.1: 1st enrichment of Iron (III) reduction of bacteria from soil sample from Antarctica 1 and 2

The recorded readings reflect the quantified ferrous iron (Fe2+) generated because of bacterial-mediated iron reduction. In the abiotic control group, where no live bacteria were present, a slight increment in iron reduction was observed over the tested days. This marginal increase in iron reduction within the abiotic control is likely attributed to abiotic factors, such as chemical or physical processes, underscoring the importance of having an abiotic baseline for comparison with the bacterial treatments. The abiotic control readings thus serve as a reference point, allowing for the distinction between biologically mediated iron reduction by bacteria and any background changes that may occur in the absence of live microbial activity.

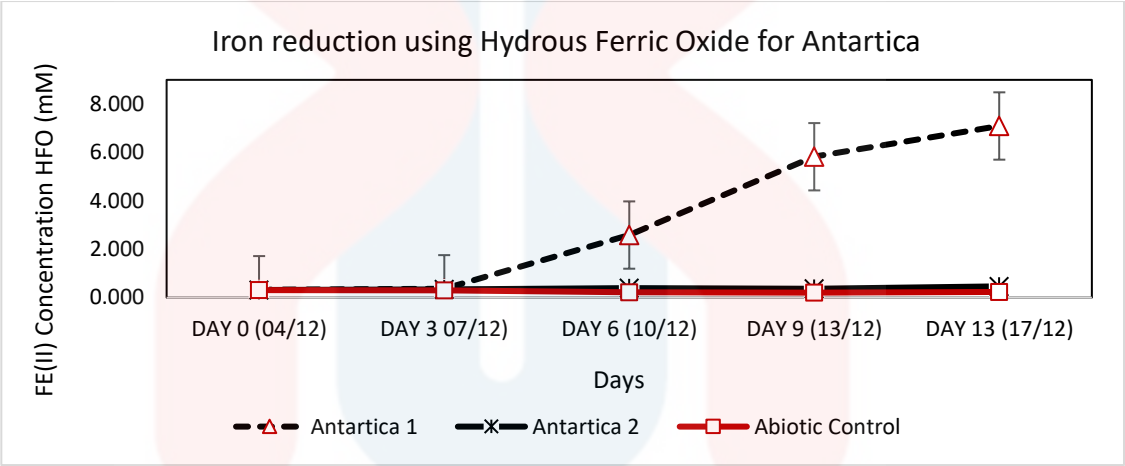


Figure 4.2: 2nd enrichment of Iron (III) reduction of bacteria from soil sample from Antarctica 1 and 2

Figure 4.2 shows the 2nd enrichment of iron (III) reduction of bacteria from soil sample from Antarctica 1 and 2 with abiotic control using yeast extract as sole electron donor and Hydrous Ferric Oxide (HFO) as the sole electron acceptor. Iron (III) reductions were indicated by the production of iron (II). Error bars represented standard deviations.

The second enrichment of iron (III) reduction reveals dynamic trends in Fe(II) concentration across the tested locations and abiotic control over the course of five days (0, 3, 6, 9, 13). In the abiotic control group, a notable decrease in Fe(II) concentration from 0.300mM on Day 3 to 0.205mM on Day 9 suggests potential abiotic processes affecting hydrous ferric oxide (HFO) reduction in the absence of live microbial activity. However, a

subsequent increase to 0.230mM on Day 13 introduces a level of complexity, hinting at the influence of fluctuating abiotic factors or other environmental dynamics.

Conversely, Antarctica 1 and Antarctica 2 present distinct patterns. Antarctica 1 experiences a substantial increase in Fe(II) concentration, rising from 0.365mM on Day 3 to 7.094mM on Day 13. This significant augmentation implies robust bacterial-mediated iron reduction, showcasing the capacity of the microbial community to enhance iron reduction over time. In contrast, Antarctica 2 exhibits a more modest fluctuation, with Fe(II) concentration values ranging from 0.340mM to 0.465mM over the same period. The stability in Antarctica 2 suggests The microbial community in Antarctica 2 may exhibit a lower diversity or distinct composition of iron-reducing bacteria (IRB) compared to Antarctica 1. This reduced microbial diversity could contribute to a more stable iron reduction process, as fewer species are actively participating in the metabolic transformation of hydrous ferric oxide (HFO) to ferrous iron (Fe(II)).

The observed numeric trends in iron reduction align with established patterns documented in the literature regarding the influence of environmental conditions on iron-reducing bacteria (IRB) activity. Microbial communities in different environments respond to variations in factors such as temperature, pH, and substrate availability, impacting the abundance and metabolic activities of IRB (D R Lovley, 1991; D. R. Lovley & Phillips, 1988). Additionally, studies emphasize the diversity of IRB in subsurface environments, where microbial communities adapt to specific conditions (Caccavo et al., 1994; Nevin & Lovley, 2002).

The distinct iron reduction rates among the tested locations corroborate findings from (Lakkana, Ashton, Hooper, Perera, & Ediriweera, 2022), who explored microbial diversity in montane soils. These studies underline the crucial role of local contexts, such as altitude, climate, and soil composition, in shaping microbial communities, including IRB. The dynamics of iron reduction Antarctica1 and 2 can be associated with the unique montane and extreme environmental conditions, respectively. This reinforces the idea that understanding IRB dynamics requires a nuanced consideration of the intricate interplay between microbial

communities and their specific habitats (Childers, Ciufo, & Lovley, 2002; Gorby & Lovley, 1991).

4.1.2 Genting Highland

Figure 4.3 shows the 1st enrichment of iron (III) reduction of bacteria from soil sample from Genting Highland point 1 and point 3 with abiotic control using yeast extract as sole electron donor and Hydrous Ferric Oxide (HFO) as the sole electron acceptor. Iron (III) reductions were indicated by the production of iron (II). Error bars represented standard deviations.

The recorded data for the abiotic control and soil samples from Genting Highlands P1 and P3 on Days 0, 4, and 7 provides valuable insights into the dynamics of iron (III) reduction, specifically the generation of ferrous iron (Fe(II)) under the influence of bacterial activity. In the abiotic control, a marginal fluctuation from 0.298mM to 0.313mM on Day 4 followed by a decrease to 0.200mM on Day 7 indicates minimal abiotic influences, establishing a baseline for comparison.

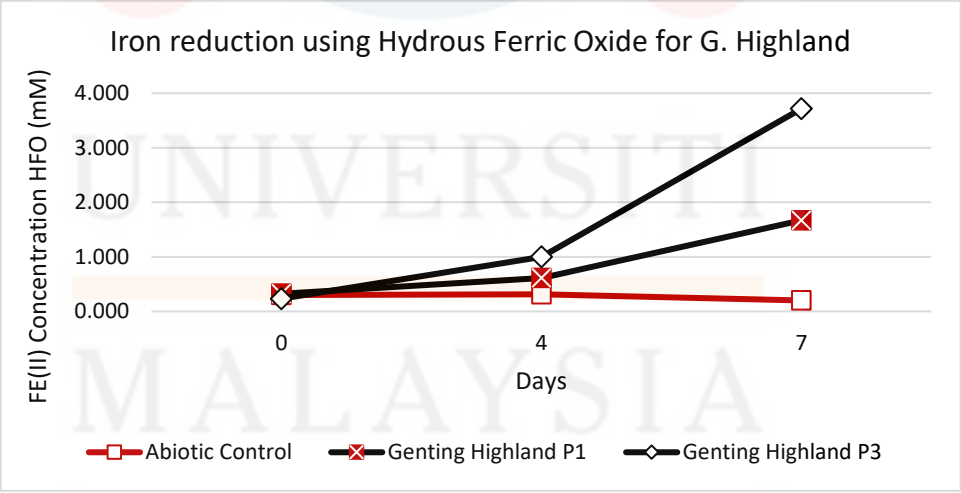


Figure 4.3: 1st enrichment of Iron (III) reduction of bacteria from soil sample from Genting Highland Point 1(P1) and Point 3(P3).

Contrastingly, Genting Highlands P1 and P3 display notable variations in iron reduction. Genting Highland P1 exhibits an increase in Fe(II) concentration from 0.323mM on Day 0 to 1.668mM on Day 7, showcasing a significant bacterial-mediated iron reduction. This upward trend suggests a thriving microbial community capable of enhancing iron reduction over the tested period. Similarly, Genting Highland P3 shows a more substantial increase from 0.233mM on Day 0 to 3.714mM on Day 7, indicating robust bacterial activity and a pronounced impact on iron (III) reduction.

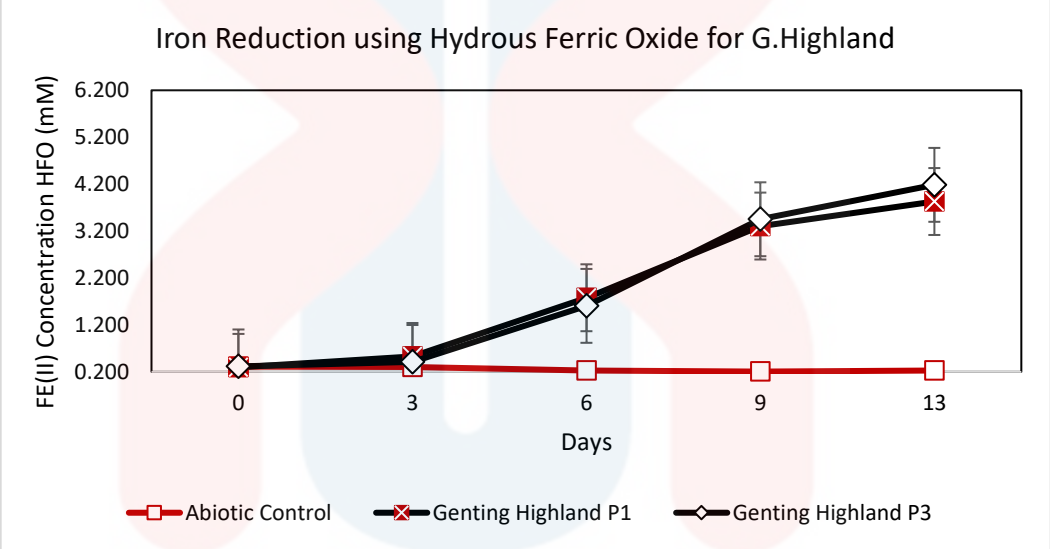


Figure 4.4: 2nd enrichment of Iron (III) reduction of bacteria from soil sample from Genting Highland Point 1(P1) and Point 3(P3).

Figure 4.4 shows the 2nd enrichment of iron (III) reduction of bacteria from soil sample from Genting Highland point 1 and point 3 with abiotic control using yeast extract as sole electron donor and Hydrous Ferric Oxide (HFO) as the sole electron acceptor. Iron (III) reductions were indicated by the production of iron (II). Error bars represented standard deviations. Examining the data from the second enrichment, the Fe(II) concentrations in the abiotic control and soil samples from Genting Highlands P1 and P3 on Days 0, 3, 6, 9, and 13 reveal intriguing trends in bacterial-mediated iron reduction.

In the abiotic control, a gradual decrease in Fe(II) concentration is observed from 0.310mM on Day 0 to 0.205mM on Day 9, followed by a slight increase to 0.230mM on Day

13. These fluctuations suggest nuanced abiotic processes at play, highlighting the importance of monitoring background changes over the experimental period.

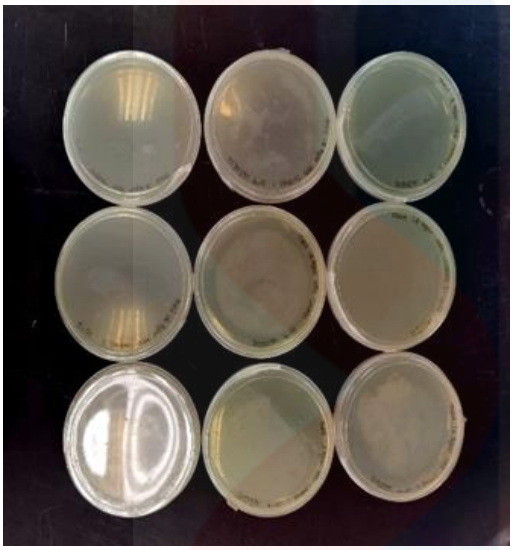
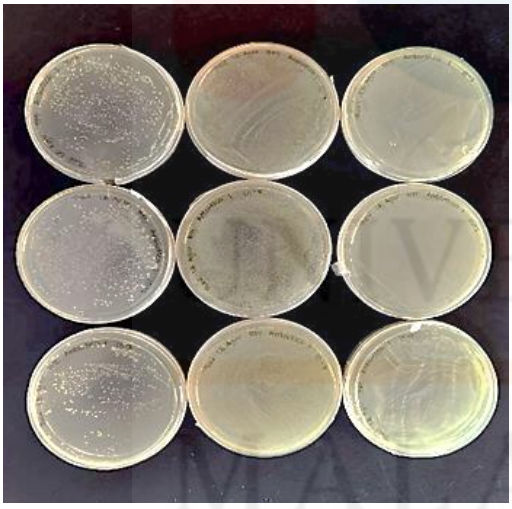
For Genting Highlands P1, there is a consistent increase in Fe(II) concentration, starting from 0.295mM on Day 0 and reaching 3.829mM on Day 13. This upward trend indicates a robust and sustained bacterial-mediated iron reduction, with the microbial community in Genting Highlands P1 demonstrating its capacity to enhance iron reduction over time.

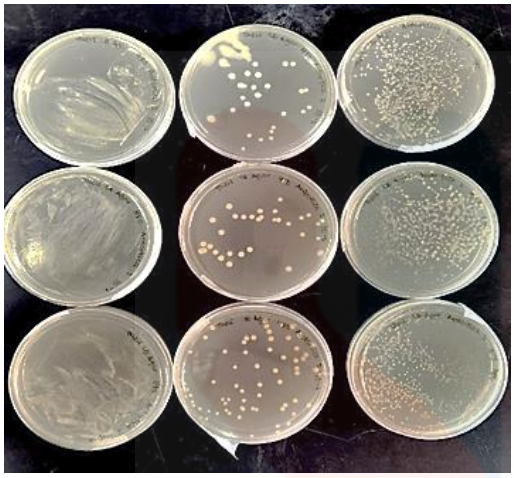
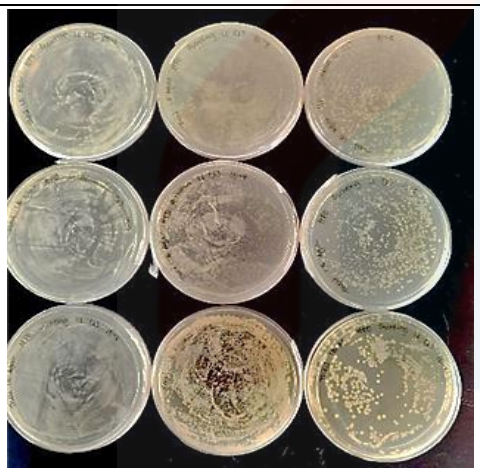
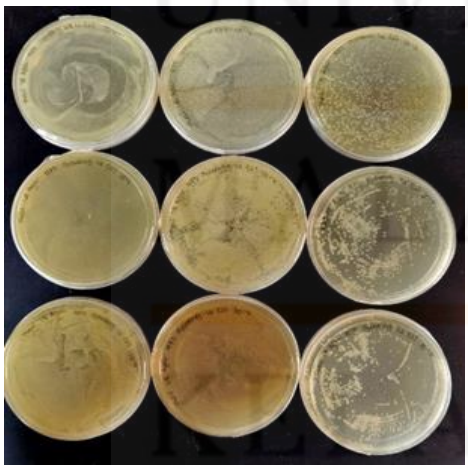
Similarly, Genting Highlands P3 displays a continuous rise in Fe(II) concentration, progressing from 0.315mM on Day 0 to 4.185mM on Day 13. This substantial increase signifies a potent bacterial activity, suggesting an efficient microbial community actively participating in iron (III) reduction throughout the experimental period.

These trends reinforce the notion that both Genting Highlands P1 and P3 harbor microbial community's adept at mediating iron reduction, showcasing their resilience and adaptability. The contrasting patterns in abiotic control further emphasize the need to discern biotic from abiotic influences when interpreting iron reduction processes. The data provides a detailed understanding of the microbial dynamics in the second enrichment, shedding light on the progressive influence of bacterial activity on iron transformations in the soil samples over the specified time frame.

Overall, the observed variations in iron reduction activities among the samples can be attributed to differences in microbial composition and environmental conditions, emphasizing the role of biological factors in driving iron reduction dynamics.

4.2 Isolation of iron reducing bacteria from Antarctica and Genting Highland

Serial Dilution Plates	Morphology
 Control (10^{-6}, $10^{-4,}$ 10^{-2})	• No colonies
 ATT 1 (10^{-6}, 10^{-4}, 10-2)	• White colonies • White yellowish colonies • Round

 ATT 2 (10^{-2}, 10^{-6}, 10^{-4})	• White yellowish colonies • Round shape
 GH P1 (1) [10^{-2}, 10^{-4}, 10^{-6}]	• White yellowish colonies • Round shape
 GH P1 (2) [10^{-2}, 10^{-4}, 10^{-6}]	• White yellowish colonies • Round shape

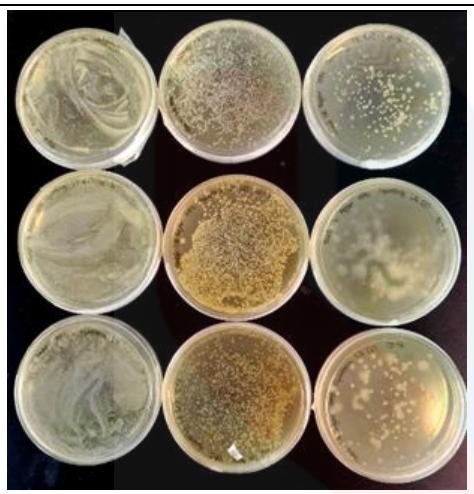
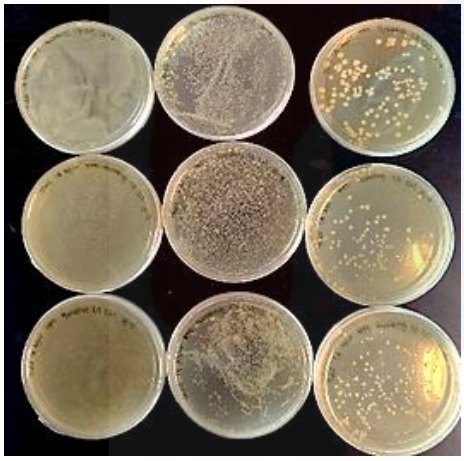
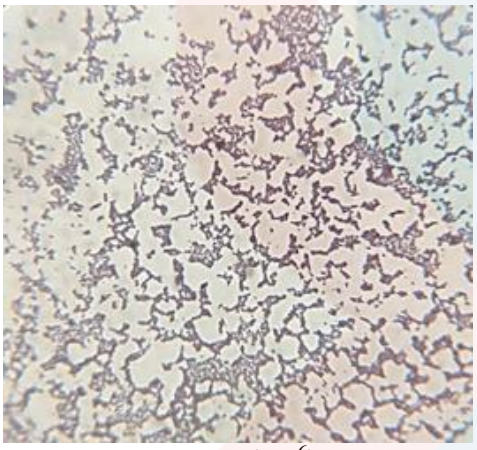
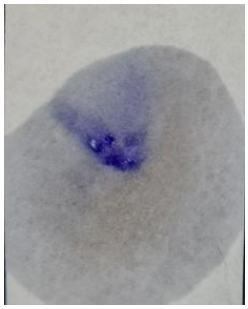
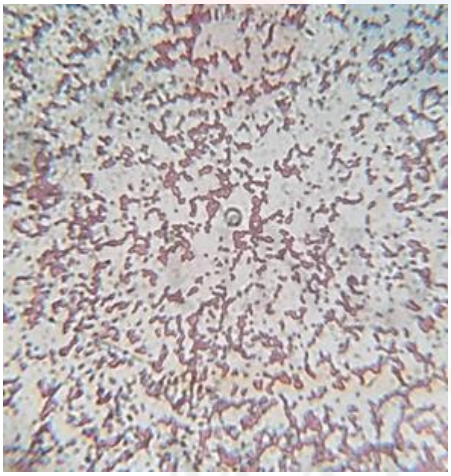
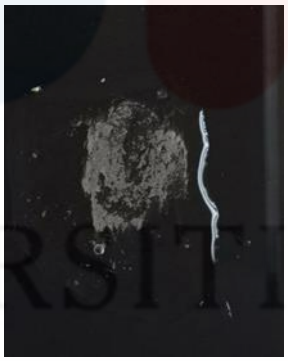
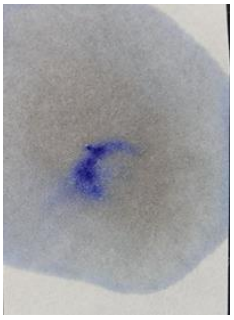
 GH P3 (1) [10^{-2}, 10^{-4}, 10^{-6}]	• White yellowish colonies • Round shape
 GH P3 (2) [10^{-2}, 10^{-4}, 10^{-6}]	• White yellowish colonies • Round shape

Table 1: Result of isolation of iron reducing bacteria from ATT, GH P1 and GH P3

4.3 Characterization of iron reducing bacteria from Antarctica and Genting highland

Gram Staining	Catalyst test	Oxidase test
 ATT 1 (10^{-6}) Gram positive	 Negative	 Positive
 ATT 2 (10^{-6}) Gram Negative	 Negative	 Positive

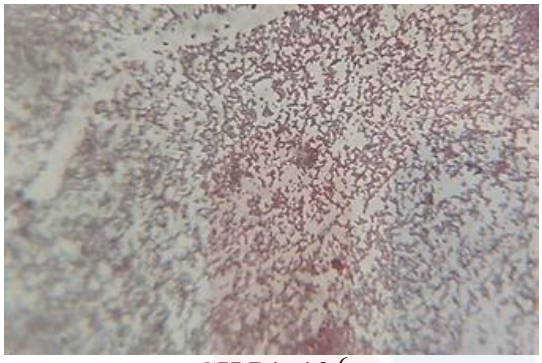
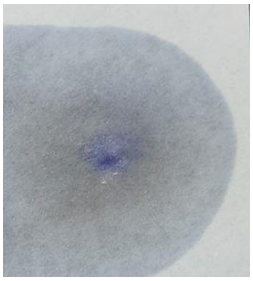
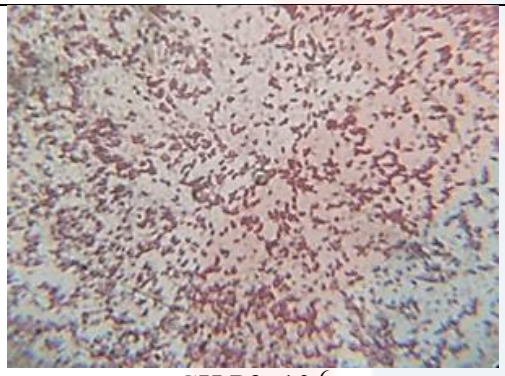
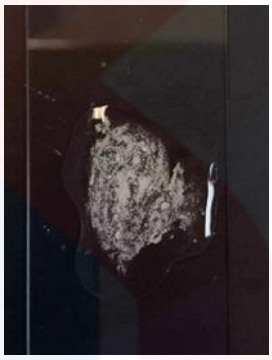
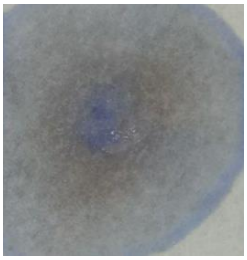
 GH P1, 10^{-6} Gram negative	 Positive	 Positive
 GH P3, 10^{-6} Gram negative	 Positive	 Positive

Table 2: Characterization of iron reducing bacteria from ATT, GH P1 and GH P3 through gram staining, catalyst, and oxidase test

The Gram staining results reveal a noteworthy distinction, with Antarctica 1 exhibiting Gram-positive bacteria and Antarctica 2, Genting Highland P1, and P3 displaying Gram-negative bacteria. Gram-positive bacteria have a thick peptidoglycan layer in their cell walls (Madigan et al., 2019). Antarctica is known for its harsh and cold climate. The thick cell wall of Gram-positive bacteria might serve as a protective barrier against environmental stresses, such as freezing temperatures. This adaptation could contribute to the survival and functionality of the microbial community in Antarctica 1. Gram-positive bacteria often exhibit diverse metabolic capabilities, allowing them to thrive in various environments. The microbial community in Antarctica 1 may rely on these metabolic adaptations to actively participate in iron reduction processes, contributing to the observed dynamics in the iron reduction experiments.

Gram-negative bacteria possess an outer membrane that can provide flexibility and versatility in adapting to diverse environmental conditions. The outer membrane acts as an additional barrier and can contribute to resistance against environmental stresses such as fluctuations in temperature, pH, and substrate availability. Antarctica 2, Genting Highland P1, and P3 represent environments with varied climates and soil compositions. Gram-negative bacteria typically exhibit a diverse range of metabolic capabilities. In the context of iron reduction, the Gram-negative bacteria in Antarctica 2, Genting Highland P1, and P3 may have evolved specific metabolic pathways that contribute to their effectiveness in mediating iron reduction processes.

Table 3 shows the result of oxidase test for iron reducing bacteria from ATT, GH P1, GH P3. All the samples turned blue. The positive results obtained in the oxidase test for both the Antarctica and Genting Highland samples hold significant implications for understanding the metabolic capabilities of their respective microbial communities. The oxidase test is commonly employed to detect the presence of cytochrome c oxidase, an enzyme involved in the electron transport chain (Artzatbanov, Müller, & Azzi, 1987). The positive outcome indicates that the microbial communities in Antarctica and Genting Highland possess the enzymatic machinery necessary for aerobic respiration, suggesting an active engagement in oxidative metabolic pathways.

In Antarctica, the positive oxidase test result aligns with the challenging environmental conditions characterized by cold temperatures and limited nutrient availability. Microorganisms thriving in such extreme environments often rely on versatile metabolic strategies to efficiently utilize available resources (Cavicchioli, 2006). The presence of cytochrome c oxidase, as indicated by the positive oxidase test, implies the ability of Antarctic microbial communities to adapt to and function in aerobic conditions, potentially contributing to nutrient cycling and ecosystem processes in cold environments (Margesin, 2017).

Similarly, in Genting Highland, a region characterized by high altitude and unique climatic conditions, the positive oxidase test result suggests that the resident microbial communities are well-equipped for aerobic metabolism. The ability to perform oxidative respiration is crucial for energy generation, and the positive oxidase test indicates that Genting Highland microorganisms

have adapted to thrive in oxygen-rich environments (Metcalf et al., 2016). This adaptability may play a role in the microbial ecology of high-altitude regions, influencing nutrient cycling and ecosystem dynamics.

The unexpected catalase test results in the anaerobic bacterial growth experiment, where Antarctica showed a negative result and Genting Highland exhibited a positive outcome, prompted careful consideration and a potential reassessment of experimental procedures. The catalase test, designed to detect the presence of the catalase enzyme catalyzing the breakdown of hydrogen peroxide into water and oxygen, typically yields negative results under anaerobic conditions, as anaerobic bacteria generally lack catalase due to adaptation to low or no oxygen environments (Khatoon, Chavan, Anokhe, & Kalia, 2022).

For the Antarctica samples, the observed negative catalase result aligned with expectations for anaerobic conditions, suggesting that microbial communities from Antarctica may indeed have exhibited anaerobic or microaerophilic metabolic traits consistent with their adaptation to the cold and oxygen-limited Antarctic environment. The negative catalase test in Antarctica samples suggests an anaerobic or microaerophilic lifestyle, aligning with the harsh, oxygen-limited conditions of the cold Antarctic environment.

Conversely, the positive catalase result for Genting Highland under anaerobic conditions was unexpected. Potential factors contributing to this anomaly, including experimental errors, contamination, or variations in bacterial metabolic activity, were considered. To troubleshoot, ensuring strict anaerobic conditions and validating the consistency of the catalase test results through repeated trials. Additionally, cross-checking for potential contamination sources and verifying the accuracy of the testing method should perform to identify and address any issues that could lead to the unexpected positive catalase outcome in Genting Highland samples under anaerobic conditions.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The comprehensive analysis encompassing iron reduction activities, serial dilution-based isolation, gram staining, and biochemical testing has yielded crucial insights into the microbial communities of Antarctica and Genting Highland. Ferrozine assay results indicate dynamic iron reduction potential in both environments. Gram staining for Antarctica 1 was Gram-positive microorganisms, while Antarctica 2 and Genting Highland indicated Gram-negative microbes. The positive oxidase test across all samples implies the presence of cytochrome c oxidase, highlighting the microbial communities' capacity for aerobic respiration. Differences in catalase test results between Genting Highland (positive) which is not align with bacterial growth condition which were incubated under anaerobic conditions and Antarctica (negative) align with distinct anaerobic conditions. In conclusion, the interpretation of iron reduction activities, Gram staining, and biochemical tests offers a comprehensive understanding of microbial communities, contributing to knowledge on extremophilic microorganisms and their adaptations. Future investigations into taxonomic composition and metabolic pathways of isolated strains will illuminate their specific roles in iron reduction processes and overall ecosystem dynamics in these unique environments.

5.2 Recommendations

I recommend refining the microbial characterization through advanced molecular techniques like 16S rRNA sequencing and metagenomic analysis for a precise taxonomic and functional understanding. Further, conduct detailed physiological and biochemical characterization of isolated strains, explore potential biotechnological applications, and investigate the broader ecosystem impact of microbial iron reduction. Incorporate transcriptomic analyses for a deeper insight into molecular mechanisms and establish a long-term monitoring program for temporal variations. Lastly, consider collaboration with other institutions to amplify the impact and scope of your research. This streamlined approach will provide a more nuanced understanding of extremophilic microorganisms and their ecological contributions.

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APPENDIX A

Figure A.1: First enrichment of serum bottles that contain 50 µl of soil samples and HFO as the electron acceptor.

Figure A.2: Second enrichment of serum bottles that contain 50 µl of soil samples and HFO as the electron acceptor.

APPENDIX B

Table B.1: OD reading iron reduction using HFO for enrichment 1

DAY	0	4	7
Abiotic Control	0.298	0.313	0.200
Genting Highland P1	0.323	0.610	1.668
Genting Highland P3	0.233	1.001	3.714
Antarctica	0.210	0.300	0.240

Table B.2: OD reading iron reduction using HFO for enrichment 2

DAY	0	3	6	9	13
Abiotic Control	0.310	0.300	0.225	0.205	0.230
Genting Highland P1	0.295	0.528	1.778	3.307	3.829
Genting Highland P3	0.315	0.410	1.606	3.452	4.185
Antarctica	0.320	0.353	1.488	3.092	3.779