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Removal of Iron Impurities from Refractory Gold Ore Using *Acidithiobacillus Ferrooxidans*

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Declaration

I declare that this thesis entitled Removal of Iron Impurities from Refractory Gold Ore Using *Acidithiobacillus Ferrooxidans* is the results of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Acidithiobacillus ferrooxidans is a chemolithoautotrophic bacterium capable of deriving energy from oxidizing ferrous iron to ferric iron, utilizing molecular oxygen as an electron acceptor. This process, known as iron oxidation, holds significance in both extracting metals from ore minerals and in the biogeochemical cycling of sulfur and iron in the environment. Microbial leaching, employing iron-oxidizing bacteria like *A. ferrooxidans*, presents a sustainable approach to reducing excessive heavy metals, such as iron (Fe), in ore minerals, thus contributing to ecosystem preservation. Traditional methods of heavy metal removal often rely on costly physical and chemical processes, posing risks to human health due to exposure to toxic chemicals like cyanide, which can lead to cyanide poisoning by inhibiting essential cellular functions. In this research, the objective is to characterize the composition of refractory gold ore and explore Fe removal from it using *A. ferrooxidans* through bioleaching, evaluating its impact on gold concentration post-process. Initially, the refractory gold ore sample's composition was analyzed via X-ray fluorescence (XRF). Subsequently, the ore sample will be subjected to a 20-day bioleaching process in a medium inoculated with the microbe. After this period, *Acidithiobacillus ferrooxidans* had successfully reduced Fe(III) to Fe(II), resulting in an increase in gold purity. Analysis using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) revealed a rise in gold concentrations from 2.009 ppm to 2.231 ppm, indicating an 11.05% increase in gold recovery. In conclusion, *A. ferrooxidans* effectively reduced Fe content in the ore sample through microbial leaching, demonstrating its potential for sustainable metal extraction processes.

Keywords: *Acidithiobaccilus ferrooxidans*, Microbial leaching, Iron-oxidizing bacteria

ABSTRAK

Acidithiobacillus ferrooxidans ialah bakteria chemolithoautotrophic yang mampu mengeluarkan tenaga daripada oksidasi besi besi kepada besi, menggunakan oksigen molekul sebagai penerima elektron. Proses ini, dikenali sebagai oksidasi besi, mempunyai makna dalam kedua-dua pengekstrakan logam daripada mineral mineral dan dalam kitaran biogeokimia sulfur dan besi dalam persekitaran. Leaching mikrobial, yang menggunakan bakteria yang mengoksidasi zat besi seperti *A. ferrooxidans*, menunjukkan pendekatan yang berkelanjutan untuk mengurangkan logam berat yang berlebihan, seperti besi (Fe), dalam mineral, dengan itu menyumbang kepada pemeliharaan ekosistem. Kaedah tradisional penghapusan logam berat sering bergantung kepada proses fizikal dan kimia yang mahal, yang menimbulkan risiko kepada kesihatan manusia disebabkan oleh pendedahan kepada bahan kimia toksik seperti cianida, yang boleh menyebabkan keracunan cianid dengan menghalang fungsi sel yang penting. Dalam penyelidikan ini, matlamatnya ialah untuk membezakan komposisi bijih emas refractory dan mengkaji penghapusan Fe daripadanya menggunakan *A. ferrooxidans* melalui bioleaching, menilai kesannya pada kepekatan emas selepas proses. Pada mulanya, komposisi sampel bijih emas refractory akan dianalisis melalui X-ray fluorescence (XRF). Selepas itu, sampel mineral akan menjalani proses bioleaching selama 20 hari dalam medium yang disuntik dengan mikroba. Selepas tempoh ini, *Acidithiobacillus ferrooxidans* akan berjaya mengurangkan Fe(III) kepada Fe(II), yang mengakibatkan peningkatan kepekatan emas. Analisis menggunakan Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) mendedahkan peningkatan kepekatan emas dari 2,009 ppm kepada 2,231 ppm, menunjukkan peningkatan 11.05% dalam pemulihan emas. Kesimpulannya, *A. ferrooxidans* secara berkesan mengurangkan kandungan Fe dalam sampel mineral melalui pelepasan mikrobial, menunjukkan potensi untuk proses ekstraksi logam yang berkelanjutan.

Kata Kunci: *Acidithiobaccilus ferrooxidans*, Bakteri yang Mengoksidasi Besi

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LIST OF ABBEREVIATIONS

A. Ferrooxidans	*Acidithiobacillus ferrooxidans*
ATP	Adenosine triphosphate
Sb	Antimony
Cd	Cadmium
CO2	Carbon dioxide
Co	Cobalt
dH20	Distilled water
Fe(iii)	Ferric iron
Fe2o3	Ferric oxide
Feco3	Ferrous carbonate
Fe(ii)	Ferrous iron
Au	Gold
Feo(oh)nh2o	Hydrated ferric oxide hydroxide
(hcl	Hydrochloric acid
In	Indium
ICP-OES	Inductively coupled plasma-optical emission spectrometry
Fe	Iron
Feass	Iron arsenic sulfide
Fes2	Iron disulphide
IOB	Iron-oxidizing bacteria
Pb	Lead
Lod	Limit of detection
Mo	Molybdenum
Ni	Nickel
No	Nitrogen oxides
OD	Optical density
Pd	Palladium
ppm	Parts per million
Pt	Platinum
Rpm	Revolutions per minute

Re	Rhenium
Rh	Rhodium
Ru	Ruthenium
Ag	Silver
So	Sulfur oxides
S	Sulphur
H2SO4	Sulphuric acid solution
Sn	Tin
Ti	Titanium
Fe3o4	Triiron tetroxide
W	Tungsten
XRF	X-ray Fluorescence
Zn	Zinc
Zr	Zirconium

LIST OF SYMBOLS

ml	Milliliters
%	Percentage
pH	Potential hydrogen ion concentration
x	Multiply
/	Divide
^{o}C	Temperature (degree Celsius)
=	Equal
>	Greater than
<	Less than
µm	Micrometer
g	Gram
µL	Microliter
L	Liter
g /L	Gram pre liter
M	Molarity
nM	Nanometer
mM	Millimolar
±	Plus and minus
σ	Standard deviations
AU	Absorbance unit

CHAPTER 1

INTRODUCTION

1.1 Background of The Study

In the current globalization era, urbanization and development in terms of social, technological, and economic are rapidly expanding. The increasing population of humans over time has made the rise in metal consumption to satisfy the needs and wants. For example, most parts of electronic devices like smartphones, laptops, households, etc. are made from metal because metal is a good efficient electric conductor. Therefore, it is important to develop effective metal extraction techniques from low-quality substances due to the limited global supplies of high-grade ores. Besides that, the expansion of the production industry will increase the waste material that is potentially hazardous to the environment. The groundwater, surface water, soil, and air can be seen to be polluted by the toxic waste from the factory (Sareh Farahani et al, 2020). Without the proper hander of waste, it may also affect the living organisms around the environment. The process of creating technology, environment, and ecosystem also needs to be given appropriate attention to keep the natural resources in the production of energy to ensure our country achieves better development in the world. Therefore, microbial leaching or bioleaching for minerals using Iron-oxidizing bacteria (IOB) is considered one of the important organisms in environmental biotechnology applications for metal recovery. Microbial leaching is often more environmentally friendly than traditional mining methods, as it does not involve the use of toxic chemicals such as cyanide.

Acidithiobacillus (A.) ferrooxidans also formerly known as Thiobacillus ferrooxidans and size up to 0.5 to 0.6 μm wide by 1.0 to 2.0 μm long with rod-shaped. *A. ferrooxidans* is commonly found in acidic environments with low pH values and optimal temperature in 30^0C, such as acid mine drainage, where it can contribute to the acidification of waterways. It is also used in the mining industry to extract metals from ores through a process known as bioleaching, where the bacterium oxidizes sulphide minerals to release metal ions. *A. ferrooxidans* has been researched for their potential use in biotechnology and bioenergy in industrial and environmental relevance. Its ability to oxidize a range of sulphur compounds, such as elemental sulphur, sulphite, and thiosulfate, has led to investigations into its potential use in sulphur

removal from industrial waste streams and biogas desulfurization. Thus, *A. ferrooxidans* is a fascinating bacterium with important ecological and industrial roles in metal biogeochemical cycling in acid environments.

A. ferrooxidans is a gram-negative and chemolithoautotrophic bacterium that is capable of obtaining energy from oxidizing ferrous iron to ferric iron using molecular oxygen as an electron acceptor. This process is known as iron oxidation and plays an important role in extracting metal from ore minerals and biogeochemical cycling of sulphur and iron in the environment. The oxidation of ferrous iron by *A. ferrooxidans* is a multi-step process that involves several enzymes and electron transport pathways. The first step is the oxidation of Fe(II) to Fe(III) by a membrane-bound cytochrome c oxidase. This reaction is coupled with the reduction of oxygen to water, generating a proton motive force that can be used to synthesize ATP. The next step is the transfer of electrons from the cytochrome c oxidase to a periplasmic rusticyanin protein, which then transfers the electrons to a membrane-bound rusticyanin protein. From there, the electrons are transferred to a cytochrome ba3 oxidase, which reduces oxygen to water, generating additional Adenosine triphosphate (ATP). Thus, *A. ferrooxidans* has a branched pathway for iron oxidation that involves the production of extracellular electron shuttle compounds, such as flavins and quinones. These compounds can transfer electrons from the bacterial cell to the mineral surface, allowing for the direct contact and oxidation of Fe2+ by molecular oxygen. The oxidation of ferrous iron by *A. ferrooxidans* is a complex process that involves multiple electron transport pathways and enzymes. This bacterium has evolved several mechanisms for oxidizing iron and other metals, allowing it to thrive in environments where these compounds are abundant.

Despite the importance of microbial metal respiration on a global scale, it is still unknown what molecules are involved in microbial iron respiration. Since gram-negative bacteria is circumneutral, the iron (II) in very soluble oxides or hydroxides cannot directly interact with the inner membrane of the electron transport chain. In order to effectively reduce solid iron (II) compounds, it is thus hypothesized that metal-reducing bacteria must use a unique approach. To study microbial iron oxidation, *A. ferrooxidans* will be used to remove iron impurities from refractory gold in this research. Understanding this process will help us understand the communities and environmental processes of these species.

1.2 Problem Statement

Microbial leaching using Iron-oxidizing bacteria (IOB) was been studied because of its capability to reduce the excessive heavy metals like iron (Fe) in ore minerals. Metal removal from waste factories is necessary for sustaining the ecosystem. Most of the methods to remove heavy metals is used by physical and chemical methods which is very costly and human health is greatly affected by toxic chemicals. Cyanide has been used widely around the world for gold and silver mining for over 125 years (Jolyn, 2016). Cyanide exposure can result in cyanide poisoning, which prevents cells from functioning normally by blocking the cytochrome c oxidase enzyme, which is essential for the synthesis of cellular energy. The cells' ability to utilize oxygen is disrupted, which results in a situation known as histotoxic hypoxia when the cells are unable to do so even though oxygen is there. Biotechnology uses microbial to treat waste like bioleaching for waste treatment is safer and eco-friendly for the environment. Whereby the need for time is to adopt a method based on green technology. Thereby, the present research can eventually assist in the removal of iron impurities from refractory gold ore using *A. ferrooxidans*.

1.3 Objectives

The purpose of the present research work is:

1) To characterize the composition of the refractory gold ore by using XRF

2) To remove the Fe from refractory gold ore using *A. ferrooxidans* via the bioleaching process

3) To investigate the influence of iron removal on the purity of aurum in the refactory gold ore after the bioleaching process

1.4 Scope of Study

The field of vision in this study investigates the use of *Acidithiobacillus ferrooxidans (A. ferrooxidans)* for the removal of iron impurities from refractory gold ore through the bioleaching process. Cultivate microbial using 9K medium to study the effectiveness of the removal of iron impurities and recovery of gold. The iron (III) oxidation activity of iron (III) reduction-deficient mutants is determined via the Ferrozine assay test.

1.5 Significance of Study

The findings of this study will contribute to the iron impurity removal activities by *A. ferrooxidans* using extracellular respiration. To carry out the study, the bacteria selected were grown together in an acidic environment. The analysis was carried out in this study by isolation and identification of the iron-reduction mutant strains. The analysis data for metabolic activities of the mutant strains also was carried out in this study. The selected *A. ferrooxidans* were then be tested against the absorbance reading to identify and determine the rate of the iron reduction activities of the bacteria in acidic conditions which is more related to the metabolic activities of the selected microorganisms. This study will create important contributions to the molecular understanding of iron reduction of *A. ferrooxidans*. Over this research, people will get to know the importance of bioleaching for minerals using Iron-oxidizing bacteria as a natural process in cleaning up and stabilization of contaminated environments.

CHAPTER 2

LITERATURE REVIEW

2.1 Composition of the refractory gold ore

The primary source of gold is extracted for the gold mineral. Refractory gold ores are difficult to process using conventional methods, such as cyanidation, due to the presence of iron impurities. These impurities can coat the gold particles and make them inaccessible to the leaching agent. The refractory gold ore mostly contains minerals like arsenopyrite, pyrite, and organic carbon (Rui Xu et al, 2020). Refractory gold ore often contains extremely fine gold particles with a diameter of less than 10 microns. These fine particles are difficult to extract because they are easily lost in the tailings during the cyanidation process. The gold particles in refractory gold ore are often encapsulated in sulfide minerals and carbonaceous materials can adsorb the cyanide ions to prevent them from reaching the gold.

2.1.1 Arsenopyrite

Arsenopyrite is an iron arsenic sulfide (FeAsS) with a grey-white colour mineral with density of 5.5 to 6.0 grams per cubic centimeter. Arsenopyrite is a toxic mineral when exposure to arsenic dust that can cause respiratory problems and skin cancer. Arsenopyrite readily weathers in nature, releasing arsenic and polluting the environment when encountering acid rain containing sulfur oxides (SO) and nitrogen oxides (NO). (Xiaonan Feng, 2021) This makes it chemically dangerous to the environment due to its toxicity.

2.1.2 Pyrite

Pyrite know as fool's gold because often mistake for gold due to its bass-yellow colour. It is an iron disulphide (FeS2) mineral that can be found in gold ore. Pyrite is the primary source of sulphur and several industries use pyrite for the production of sulfuric acid.

2.1.3 Carbonaceous matters

Carbonaceous matter is a general term for organic matter that is found in rocks and sediments. It is composed of carbon, hydrogen, oxygen, and nitrogen, and can be found in a variety of forms, including coal, peat, and kerogen. Carbonaceous matter is formed from the decomposition of plant and animal matter. When these organisms die, they are buried in sediments. Over time, the heat and pressure of the Earth's crust transform the organic matter into carbonaceous matter. Carbonaceous matter can be used in the production of steel, cement, and other industrial products.

2.2 X-ray Fluorescence (XRF)

XRF is a versatile and powerful analytical technique that provides rapid and accurate results for elemental analysis across diverse fields, including chemistry, geology, archaeology, and environmental science. Its non-destructive nature makes it particularly valuable for analyzing valuable or sensitive samples. XRF involves the use of X-rays to excite the inner-shell electrons of atoms in a sample. When high-energy X-rays bombard the sample, causes inner-shell ionization and electrons are ejected from the atoms. As a result of inner-shell ionization, electrons from higher energy levels fall to fill the vacancies in the inner shells. This process leads to the emission of characteristic X-rays that are unique to each element. Lastly, the characteristic X-rays emitted by the sample are then detected and analyzed. The energy and intensity of these X-rays are used to identify and quantify the elements present in the sample.

2.3 Iron oxidation bacterial

Iron-oxidizing bacteria alternatively known as iron bacteria are chemotrophic microorganisms that produce energy by oxidizing dissolved iron. They are known to thrive and multiply in waters with iron concentrations as low as 0.1 mg/L and the oxidation must be carried out with at least 0.3 ppm of dissolved oxygen (Andrews et al, 2013). The first anaerobic metabolism inside the iron anaerobic oxidation metabolism to be characterised was the anoxygenic phototrophic iron oxidation. The Calvin-Benson-Bassam cycle is used by photoferrotrophic bacteria to convert CO2 into biomass in a neutrophilic environment range of pH 5.5 to 7.2. Fe(III) oxides as a waste product are produced from the concentrate as a mineral.

2.4 *Acidithiobacillus ferrooxidans (A. ferrooxidans)*

Acidithiobacillus ferrooxidans was discovered by Colmer, Temple, and Hinkle in 1950 and has become a commercially relevant bacterium in the field of biohydrometallurgy in the leaching of sulphide ores. *A. ferrooxidans'* discovery sparked the growth of "biohydrometallurgy," which covers all facets of microbial mediated metal extraction from solid wastes and acid mine drainage. (Colmer et al, 1950). It is well known that many bacterial organisms utilize hydrogen as an electron donor to boost their development (Sabrina et al, 2013). *A. ferrooxidans* is a chemolithoautotrophic bacteria that can grow using both elemental sulphur (S) and the oxidation of ferrous iron as a source of energy because of its metabolism to absorb light and use chemical reaction to oxide the inorganic substrate as the source of energy for cell biosynthesis. Its cellular architecture is club or rod geometries tied to its ability to obtain energy sources. *A. ferrooxidans* become used to survive in an environment with temperatures as high as 97.6°C because of its thermophilic ability to resist heat. Thus, it also evolved into acidophilic and neutrophilic microbial that can adapt in low pH value around 0.5-6.0 (Sriaporn et al, 2021).

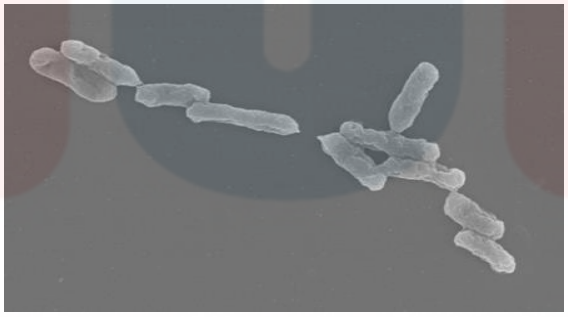

Figure 2.1: Picture of *A. ferrooxidans* under microscopy

2.5 Dissimilatory iron oxidation mechanisms

Understanding the dissimilatory iron reduction mechanism is essential to use IOB in environmental remediation successfully. IOB reacts with solid mineral surfaces, making a prior study on bacterial respiration on soluble electron acceptors inapplicable. What mechanisms IOB have developed to use Fe (III) oxyhydroxides as a source of oxygen is thus a major topic. Finding out whether iron oxidation is surface- or transport-limited is also crucial since it is a process that occurs on the surface of minerals. In a reaction that is surface restricted, the reaction on the mineral surface limits the rate, but in a reaction that is transport limited, the rate is limited by the movement of reactants for surface reactions. It is still not fully understood how iron oxidation works.

2.6 Microbial leaching

Bioleaching or bacterial leaching is a biohydrometallurgical process that relies on the ability of biological agents such as iron oxidizing bacteria or iron reducing bacteria to transform solid compounds into soluble and recovered purity of gold in the refractory gold ore by the extraction of metals impurity. Bioleaching is a process described as the dissolution of metals from their mineral sources by certain naturally occurring microorganisms (Debaraj Mishra et al., 2005). These microorganisms employ various mechanisms to oxidize metal sulfides, including direct and indirect mechanisms. Direct mechanisms involve the oxidation of metal sulfides by microorganisms through the action of enzymes, while indirect mechanisms involve the production of metabolites that oxidize metal sulfides. Additionally, bioleaching is key in the sustainable recovery of metals from solid matrices, especially considering the enormous amounts of waste produced every year (Ramirez-Carmona, M et al., 2023). Hence, bioleaching offers several advantages over conventional methods of metal extraction, including lower energy consumption, reduced environmental impact, and enhanced selectivity. It operates under mild conditions, avoiding the need for high temperatures and pressures, and generates fewer harmful by-products.

2.7 Element Properties of Iron

Iron is the second most abundant metal in the crust of the Earth after aluminium and ranks fourth among all elements, behind silicon, oxygen, and aluminium. Iron is a chemical element with the symbol Fe and it has 26 of nuclear charge number. A large number of minerals contain iron in combination with other elements; the most valuable of these are siderite (ferrous carbonate, $FeCO_3$), hematite (ferric oxide, Fe_2O_3), limonite (hydrated ferric oxide hydroxide, $FeO(OH)nH_2O$), and magnetite (triiron tetroxide, Fe_3O_4). The average of iron concentration in igneous rocks is 5%. Iron also available in our daily product in heme and non-heme type. Only animal flesh contains heme, including poultry, meat, and fish. Hence, non-heme iron is contained in plant foods like nuts, legumes, whole grains, and leafy greens.

2.8 Factors that will affect the microbial leaching process

Even though *A. ferrooxidans* have the ability to grow in the harsh condition by reason of their thermophilic metabolism which can chemical stability in the membrane lipids at high temperatures. However, several factors can affect the efficiency of the microbial leaching process such as nutrients in the medium, pH value, temperature, oxygen, and carbon dioxide supply (Sarkodie et al, 2022). All these factors are called physicochemical factors that can influence the synthesis of the necessary metabolites as well as the microorganisms' growing circumstances.

2.8.1 pH value

The pH of the leaching environment affects microbial activity and metal solubility. Acidic conditions (pH below 3) are usually favorable for bioleaching, as they promote the dissolution of metal sulfides. The pH needs to be carefully controlled to maintain optimal conditions for the microbial species involved.

2.8.2 Temperature

Temperature influences microbial growth rates and enzymatic activity. Different microbial species have specific temperature ranges in which they function optimally. Typically, bioleaching is conducted at temperatures ranging from 30°C to 45°C, depending on the specific microorganisms used.

2.8.3 Nutrient in the Medium

Microbes involved in bioleaching require essential nutrients such as carbon, nitrogen, phosphorus, and trace elements to support their growth and metabolic activities. The availability and balance of these nutrients impact microbial growth rates and, subsequently, the leaching efficiency.

2.8.4 Oxygen Supply

Microbial leaching is an oxidative process that requires an adequate supply of oxygen. Oxygen availability affects microbial metabolism and the rate of metal oxidation. Efficient aeration or agitation systems are employed to ensure sufficient oxygen transfer into the leaching system.

2.9 Microwave Digestion

Microwave digestion offers advantages such as speed, efficiency, and the ability to handle a variety of sample types. Consequently, it is a sample preparation technique used in analytical chemistry to break down complex matrices, such as organic and inorganic samples, for subsequent analysis (Garitta, J. A. et al., 2021). This process involves exposing the sample to microwave radiation in the presence of a mixture of strong acids. The goal is to facilitate the decomposition of the sample into a more manageable form for analysis. Microwave digestion is widely used for the preparation of geological samples, such as soil, ores, rocks, and sludge, for elemental survey. However, proper safety precautions must be observed due to the use of strong acids and the potential for pressure build-up during the digestion process.

2.10 Inductively coupled plasma-optical emission spectrometry (ICP-OES)

ICP-OES is a powerful and versatile analytical technique that allows for the rapid and accurate determination of the elemental composition of diverse samples. Its high sensitivity and ability to analyse multiple elements simultaneously make it invaluable in various scientific and industrial applications (Mindy Levine, 2021). The sample is typically introduced into the instrument as a liquid, often in the form of a acid solution with suitable solvet. This solution can be aspirated into the system using a nebulizer. The sample is then introduced into an inductively coupled plasma torch, where it is rapidly heated to a temperature of around 10,000 K. This high-temperature environment causes the atoms in the mist to be excited, meaning that they absorb energy and move to a higher energy state. This is a process known as atomization. The excited ions or atoms in the plasma return to their ground state, emitting characteristic wavelengths of light during this process. The emitted light is then passed through a spectrometer, which disperses the light into its component wavelengths. A detector measures the intensity of the emitted light at specific wavelengths corresponding to the elements of interest. The intensity of the emitted light is directly proportional to the concentration of the elements in the sample. Finally, The data obtained from the detector are processed using specialized software to quantify the concentrations of elements in the sample.

CHAPTER 3

3.0 RESEARCH METHODOLOGY

3.1 Material

In this experiment, the 9K medium has the important function to provide energy for *A. ferrooxidans* to grow. 9K medium composition of Ammonium sulphate, Dipotassium phosphate, Magnesium sulphate, Potassium chloride, Calcium nitrate, Iron(II) sulphate heptahydrate, agar powder, and distilled water. Sulphuric acid solution (H2SO4) and sodium hydroxide solution (NaOH)will be used to control pH value.

3.2 Apparatus and Equipment

The apparatus and equipment will be needed in this research such as measuring cylinder, spatula, hot plate, glove, autoclaved, media bottles, refrigerator, pH meter, 250 mL conical flasks, incubator shaker, X-ray diffraction Spectrometry, microscope, spectrophotometer, cuvette, and micropipette.

3.3 Raw material

In this study, ore minerals will be applied as sample in this research. The refractory gold ore will be collected from Gua Musang, Kelantan and the Ferron oxidant will be brought from the factory.

3.4 Methods

3.4.1 Preparation of the refractory gold ore sample

X-ray fluorescence analyzer (XRF) was used to analyse the concentration of the elements in the refractory gold ore sample before recovery. The data was collected for researcher observation. After that, the refractory gold ores were grounded into small sizes using a crusher for microbial to easily consume and sieved with RETSCH Sieve Shaker AS 200 until size < 32 μm.

3.4.2 Microorganism and Cultivation

A strain of *A. ferrooxidans* was conserved in the laboratory using a low temperature of the refrigerator and was used for these experiments research. Next, the microbial shall be cultivated in a growth medium.

3.4.3 Preparation of culture medium

9K medium was prepared in 1L of solution with two parts. First, Solution A was make ready in 700 mL with Ammonium sulfate 3 g /L, Dipotassium phosphate 0.5 g /L, Magnesium sulfate 0.5 g /L, Potassium chloride 0.1 g /L, Calcium nitrate 0.01 g /L. Then, solution B contained Iron(II) sulfate heptahydrate 44.7 g /L and 1% agar powder in 300 mL. The pH value of the medium was adjusted using diluted hydrochloric acid and sodium hydroxide solution around 1.8-2.0.

3.4.4 Microbial leaching

The refractory gold sample was transferred into the conical flask of 50ml of 9k medium with 1ml of inoculum *A. ferrooxidans* and each conical flask had different sample size of 0.5 g, 1 g, 1.5 g, and 2 g refractory gold ore. Afterward, the microorganisms and the solution with be placed in the incubator for 20 days of bio-oxidation at 30^{o}C with 180 rpm to carry out the bioleaching process. The concentration of iron (III) will be measured every 4 days using ferrozine assy. In the whole process, a control sample was also prepared in the incubator to ensure the validity and reliability of the bioleaching process.

3.4.5 Analyzation of Fe (III) oxidation activity via Ferrozine assay

Iron concentration was analysed using Ferrozine essay. First, 200 µL of samples from the flask shall be extracted into 2 mL of microcentrifuge tubes with 1 mL of 0.5 M hydrochloric acid (HCl) and left for 20 minutes. The maintenance of low pH was to stop Fe (II) from oxidizing and dissolving Fe (III) particles to release Fe (II) that has been absorbed in Fe (III) particles. Next, 50 µL of the sample will be transferred to new microcentrifuge tubes with 950 µL of Ferrozine. Absorbance readings were recorded and measured by using a spectrophotometer at 562 nm and adjusted blank at zero with dH2O. Afterward, the concentration of produced iron (II) can be measured minus the final optical density reading of the sample with 0.016 then divided by 0.1999. (Wee, 2014)

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

3.4.6 Separation process

After the microbial leaching for 20 days, the refractory gold ore at the bottom layer of the solution will be separated by using centrifugation, which is a separation method of solid-liquid extraction. Each sample will be fill in the different 50 ml of falcon tube and were put in centrifuge machine for 10 minutes at 5000 rev/min. The supernatant need to be removed. Next, the pallet will be place on the glass petri dish and dried at ± 100°C in the oven to remove moisture from the solid sample. Afterward, the dried samples will be recovered using the microwave digestion method to dissolve certain heavy metal.

3.4.7 Total digestion using microwave digester

The sample will be weighed precisely in the sample cup using laboratory balances and placed in the vessel. Next, add the appropriate volume and concentration of acids to the vessel. The acid regent are mix of nitric acid, hydrochloric acid, hydrofluoric acid, and sulfuric acid with volume ratio of 4:3:1.5:1.5. Then, close the vessel with the pressure seal and DPC cap. Afterward, the digestion vessel will be place in the microwave cavity and set the digestion program to control the temperature, pressure, and time of the digestion process. After the digestion is complete, allow the vessel to cool down before proceeding next step.

3.4.8 Analyzation of gold using ICP-OES

The vessel need to be open carefully and pour the solution in the Teflon beaker to heat the solution with 0.5M nitric acid until the solution is near-dryness and colourless. The sample then needs to be cooled to room temperature and filtered using filter paper or 0.45 μm syringe filter. The sample will be poured in the 50mL centrifuge tube and added the 0.5 M nitric acid up until 50 mL of volume. The concentration of Au in the samples was analysed using ICP-OES.

Chapter 4

RESULT AND DISCUSSION

4.1 Analysis composition of the refectory gold ore by XRF

S1 TITAN Handheld XRF Analyzer is a lightweight and rugged X-ray fluorescence analyzer designed for fast analysis speed and exceptional accuracy. This experiment aimed to sort the minerals' composition in the refractory gold ore samples. The S1 TITAN is an ideal solution for on-site elemental analysis, offering easy operation and portability for various applications. The table below shows that iron is the most filled element in refractory gold ore samples, with approximately 66.739% of the samples containing iron elements. The second highest value in the sample is rhodium about 9.288%. The element gold in the refractory gold ore sample was very least about 0.469%. The LOD in the XDF analysis table stands for the limit of detection (Badlaa, C., & Wewers, F., 2020). The LOD represents the lowest concentration of a specific element that can be reliably detected by the XRF instrument, such as carbon monoxide, nickel, molybdenum, silver, cadmium, antimony, tungsten, platinum, lead, and rhenium. Besides that, the notation "±2 standard deviations " refers to indicating the confidence range around the mean of a sample's XRF data. In X-ray techniques, small variations in the sample can affect the precise position of peaks, so reporting a range helps convey the level of uncertainty associated with the measured angles.

Table 4.1: Elements found in the refractory gold ore sample before bioleaching by *A. ferrooxidans*

Element/Compound	%	±2σ	Element/Compound	%	±2σ
Ag	<LOD	0.356	Pt	<LOD	0.105
Au	0.469	0.165	Re	<LOD	0.134
Cd	<LOD	0.403	Rh	9.288	0.312
Co	<LOD	0.128	Ru	5.017	0.178
Fe	66.739	0.692	Sn	1.620	0.640
In	6.355	0.565	Sb	<LOD	1.097
Mo	<LOD	0.064	Ti	2.539	0.209
Ni	<LOD	0.060	W	<LOD	0.102
Pb	<LOD	0.249	Zn	2.242	0.071
Pd	3.589	0.533	Zr	2.144	0.093

4.2 Removal of Fe via bioleaching process using *A. ferrooxidans*

4.2.1 Bacterial growth curve

Before starting the bioleaching process, the *A. ferrooxidans* need to be cultured in a solution of 9k medium to adapt to the acid environment with a time observation of 20 days. Furthermore, the culture will be extracted at the stationary phase where a maximum cell density in liquid culture is reached. This can increase the efficiency of the microbial leaching of refractory gold ore. After mixing the inoculated *A. ferrooxidans* with 9k broth medium, the optical density (OD) was monitored by Thermo Scientific GENESYS 20 Spectrophotometer at a wavelength of 600 nm over time to understand the bacterial growth curve. At the same time, blank control was also prepared with 9k medium without adding *A. ferrooxidans*. The purpose of the control setup is to help identify contamination and act as a reference point to compare the experimental results. The OD reading of the blank control from the first day to the last day was around 0.021 to 0.035 AU. This outcome demonstrates there is no contamination during the culturing of bacteria.

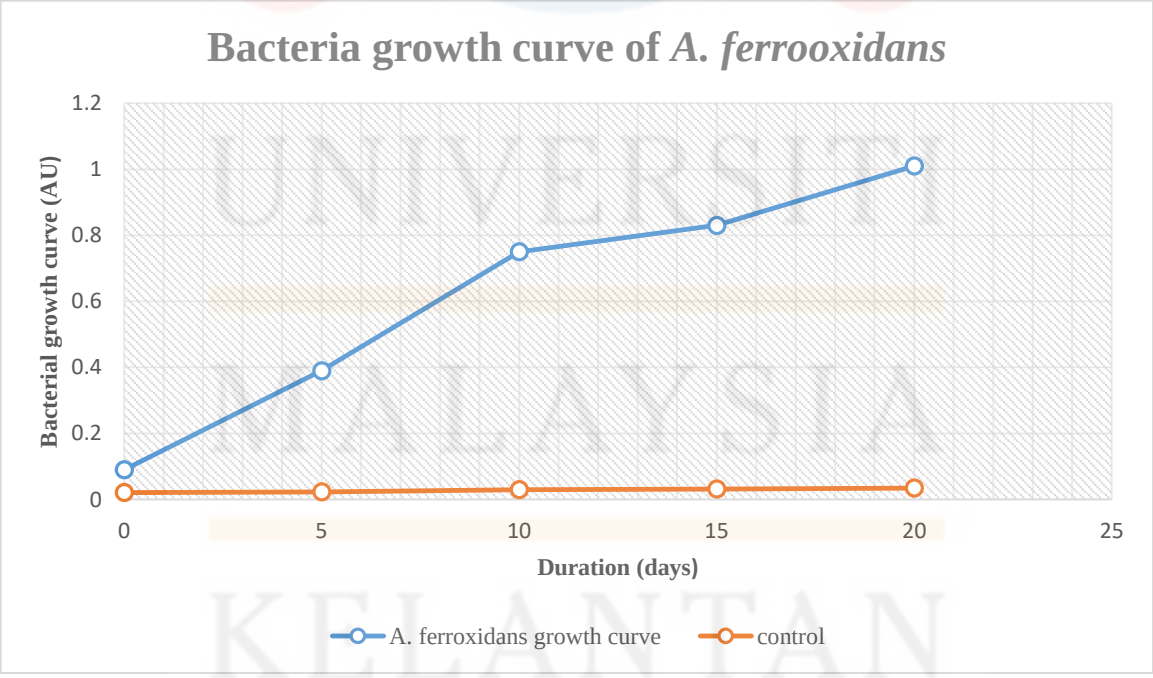

Figure 4.1: Bacteria growth curve of *A. ferrooxidans* in 9k medium broth

The OD reading of bacterial growth was 0.09 Absorbance Units (AU) on the first day. The next OD reading after five days was 0.39 AU. This indicates the bacterial cells enter the exponential phase. The exponential phase is critical in the growth of any bacterial population, including those cultures of iron-oxidizing bacteria (IOB) that reside in acidic environments. During this phase, the number of bacteria doubles at a constant rate, leading to incredibly rapid population increases under optimal conditions. These bacteria have access to sufficient nutrients, iron sources, and optimal acidity levels, enabling them to efficiently convert Fe(II) to Fe(III) while generating energy. Then, the third and fourth OD reading results were 0.75 AU and 0.83 AU. Eventually, bacterial cell growth reaches the stationary phase, where the rate of cell division becomes equal to the rate of cell death and leads to a relatively constant population density despite ongoing metabolic activities. On the 20^{th} day, the OD reading was 1.01 AU, and decided to extract 1mL of the inoculum bacteria culture into each sample to run the bioleaching process.

4.2.2 The process of Fe oxidation using *A. ferrooxidans*

A. ferrooxidans is a type of bacteria that is capable of oxidizing iron and sulfur compounds present in minerals, which release metal ions that are then solubilized in water. *A. ferroxidans* was used in this experiment to investigate the ability to extract metals from the ores and increase the gold purity percentage. The process involves placing the ore sample in a flask with a nutrient solution that contains the bacteria. In order to calculate the oxidation of iron during 20 days, Figure 3 displays a reading from the Ferrozine assay at 562 nm using a spectrometer. In the absence of bacterial strains, each sample contains a different concentration of ore sample of 0.5g, 1.0g, 1.5g, 2g, and a control shown in Figure 3.

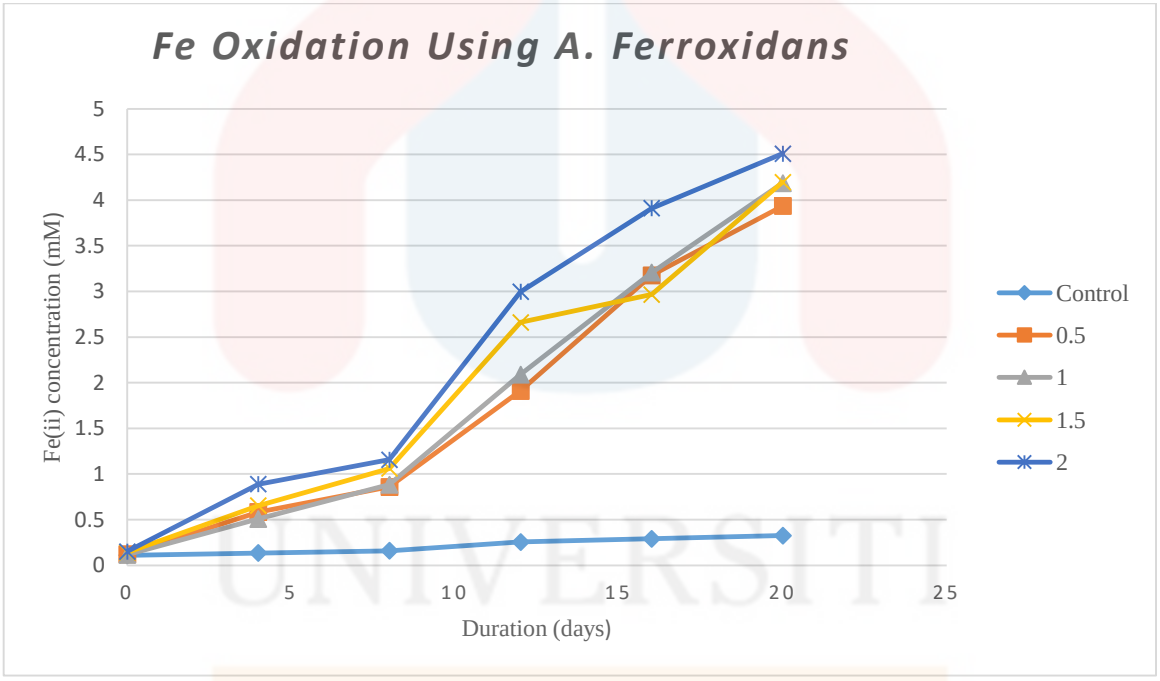

Figure 4.2: *A. ferrooxidans* iron oxidation with different concentration of ore samples of 0.5g, 1.0g, 1.5g, 2g, and Control for 20 days of bioleaching

The Ferrozine assay is a widely used colourimetric method for quantifying the concentration of ferrous iron in a solution. (Jeongdae Im et al., 2013) The pH range for the Ferrozine assay is also an important consideration for its accurate and reliable performance because it is a very active and sensitive chemical compound when mixed with other compounds. The purple colour was created by the reaction of the ferrous iron with the ferrozine reagent and bind the iron particles in the solution. Due to the high absorbance of the purple-coloured complex, iron concentrations as low as 0.05 μM can be detected. The darker purple colour in the solution is indicative of a larger concentration of ferrous iron. Essentially, the absorbance of the ferrous-ferrozine complex at 562 nm allows for the determination of iron concentration in the solution. The graph illustrates the concentration of iron in the sample after it has been transformed by the *A. ferrooxidans* from solid metal waste into a form that can be extracted and dissolved into a solution. In addition, it was also observed that the colour of the sample changed from grey to dark yellow-green within 20 days after the bioleaching experiment.

As can be seen in Figure 4.2, varying sample concentrations led to varying ferrous concentrations. Low readings of Fe (II) content in the control medium indicated no reaction, proving that the presence of bacterial strains was necessary for Fe reduction. According to observation, Fe oxidation is greater in the treatment for the 2.0g of refractory gold ore than in the other three sample concentrations. It has been show that the high sample concentration resulted in a high Fe (III) reduction and the pH value of the medium broth stays in the consistent range around 2.0 to 2.2. The highest yields of metal extraction can be acquired once the parameters for bioleaching match the ideal conditions for bacterial growth. The fact that *A. ferrooxidans* has the ability to grow by means of Fe (II) oxidation and Fe (III) reduction, which relies on the mechanism of cytochrome transfers the electrons to produce ATP. (Yang M, et al, 2020)

4.3 Fe and Au element analysis by ICP-OES

In this section, different element concentrations of the sample were tested after receiving bioleaching as a treatment. The untreated sample is the original sample. Before the samples were running the analysis test, the sample needed to be recovered by undergoing some recovery steps such as centrifugation and drying. Afterwards, the microwave digestion technique is used to remove organic and inorganic particle from the sample by using high heat, pressure and strong acid. 0.25 g of each treated and untreated ore sample was placed in the vessel for the microwave digestion process. The solution resulting from the digestion is yellowish in colour with suspended solid particles until it becomes a transparent solution with 0.5 M nitric acid.

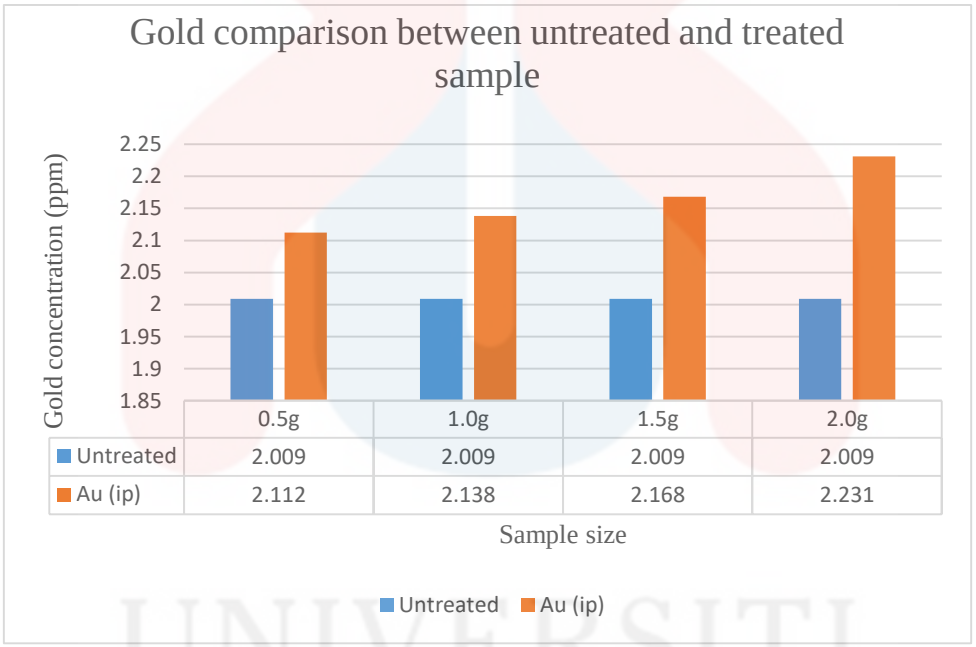

Figure 4.3: Comparison purity of gold between untreated and treated sample

Figure 4.3 shows the gold element concentration was examined using ICP-OES. All treated samples had a higher gold concentration of gold than the untreated sample after 20 days of bioleaching. The untreated sample without bioleaching by *A. ferrooxidans* only had 2.009 ppm of gold concentration. Additionally, 2.112 ppm of gold concentration with 5.13% gold recovery from 0.5g of sample size and 2.138 of gold concentration with 6.42% gold recovery from 1g of sample size. The 1.5g sample size also had 2.168 ppm of gold content with 7.91% of gold recovery. 2.0g of sample size had the highest gold concentration with 2.231 ppm and 11.05% of gold recovery.

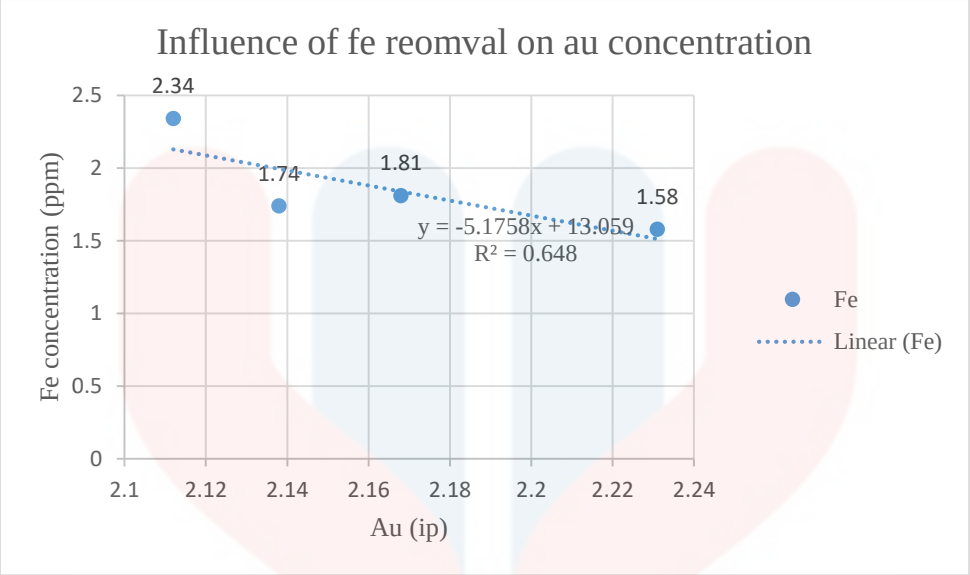

Figure 4.4: The influence of Fe removal on Au concentration following the bioleaching process

ICP-OES analysis was conducted to measure the concentration of Fe in the untreated and treated samples, demonstrating variations in Fe concentration. The untreated sample contained 4.1 ppm of Fe concentration, while the 0.5g of the treated sample had 2.34 ppm of Fe concentration. Next, the 1.0g, 1.5g and 2.0g of treated samples contained 1.74 ppm, 1.81 ppm, and 1.58 ppm of Fe concentration. From Figure 4.4, the Fe content in the treated samples had less concentration than the untreated sample. This indicates the higher the size of the refractory gold ore sample, the lower the Fe concentration for each refractory gold ore sample. This had to be related to the ability of microbial leaching by *A. ferrooxidans* to remove Fe impurity from the refractory gold ore. Therefore, as the removal of Fe impurities from refractory gold ores increases, the quality of the gold in the ores is expected to improve.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The purpose of this research project was to remove the Fe from refractory gold ore using *A. ferrooxidans* via the bioleaching process and analyse the different concentrations of elements before and after treatment. By using the X-Ray Diffraction (XRD) to analyse the concentration of elements in the refractory gold ore from Gua Musang, Kelantan analyze, showed that the percentage of Fe concentration in the ore sample is very high about 65% and the presence of pyrite and arsenopyrite in sample. The higher the size of the refractory gold ore sample, the more Fe content was removed throughout bioleaching. Based on the result, the Fe content in the ore sample was reduced by *A. ferrooxidans* after the microbial leaching process. This is due to the ability of *A. ferrooxidans* to obtain energy from the oxidation of ferrous iron and also utilize elemental sulfur to grow. (Mengran Yang et al., 2020) Consequently, the gold concentration increased from 2.009 ppm to 2.100 ppm after bioleaching. This research concluded that the *A. ferrooxidans* improved the gold purity by recovery throughout microbial leaching.

5.2 Recommendations

The study aimed to focus on microbial leaching refractory gold ore using *A. ferrooxidans* under a low pH environment. The environment or physical circumstance is crucial for bacterial growth. Therefore, further research can be carried out by using different pH value mediums to increase the growth of the microbe and the effectiveness of bioleaching. The experiment also can be conducted by using a suitable assay method to detect sulfur concentration during bioleaching by *A. ferrooxidans*. This is because *A. ferrooxidans* able to efficiently oxidize several reduced inorganic sulfur compounds (RISCs) under extreme conditions for their autotrophic growth. (Juan J. R. Coque et al, 2023) A thorough knowledge of this method will also shed light on the environmental dynamics and communities that *A. ferrooxidans* inhabit. Furthermore, the size of the ore sample should be increased to have more samples to test the element analysis and improve the surface area available for microbial action.

REFERENCES

Hedrich, S., & Johnson, D. B. (2013). Aerobic and anaerobic oxidation of hydrogen by acidophilic bacteria. FEMS Microbiology Letters. https://doi.org/10.1111/1574-6968.12290

Colmer, A. R., Temple, K. L., & Hinkle, M. E. (1950). An iron-oxidizing bacterium from the acid drainage of some bituminous coal mines. Journal of Bacteriology, 59(3), 317–328. https://doi.org/10.1128/jb.59.3.317-328.1950

Sriaporn, C., Campbell, K. A., Van Kranendonk, M. J., & Handley, K. M. (2021). Genomic adaptations enabling Acidithiobacillus distribution across wide-ranging hot spring temperatures and PHS. Microbiome, 9(1). https://doi.org/10.1186/s40168-021-01090-1

Andrews, S., Norton, I., Salunkhe, A. S., Goodluck, H., Aly, W. S., Mourad-Agha, H., & Cornelis, P. (2013). Control of iron metabolism in bacteria. Metal Ions in Life Sciences, 203–239. https://doi.org/10.1007/978-94-007-5561-1_7

Farahani S, Akhavan Sepahi A, Shojaosadati SA, Hosseini F. (2020). Bio-removal of Heavy Metals using Iron-oxidizing Bacteria: A Novel Approach in Environmental Biotechnology. Iran J Pharm Res., 421-429. https://doi.org/10.22037/ijpr.2019.112474.13779

Xu, R., Li, Q., Meng, F., Yang, Y., Xu, B., Yin, H., & Jiang, T. (2020). Bio-oxidation of a double refractory gold ore and investigation of preg-robbing of gold from thiourea solution. Metals, 10(9), 1216. https://doi.org/10.3390/met10091216

Cai, G., Ebrahimi, M., Zheng, G., Kaksonen, A. H., Morris, C., O'Hara, I. M., & Zhang, Z. (2021). Effect of ferrous iron loading on dewaterability, heavy metal removal and bacterial community of digested sludge by *Acidithiobacillus Ferrooxidans*. Journal of Environmental Management, 295, 113114. https://doi.org/10.1016/j.jenvman.2021.113114

Sarkodie, E. K., Jiang, L., Li, K., Yang, J., Guo, Z., Shi, J., Deng, Y., Liu, H., Jiang, H., Liang, Y., Yin, H., & Liu, X. (2022). A review on the bioleaching of toxic metal(loid)s from contaminated soil: Insight into the mechanism of action and the role of influencing factors. Frontiers in Microbiology, 13. https://doi.org/10.3389/fmicb.2022.1049277

Jo-Lyn, N. (2016, June 7). Is this Pahang kampung getting sick from gold mining? we investigate... CILISOS. https://cilisos.my/is-this-unknown-gold-mine-in-pahang-poisoning-villagers-with-cyanide/

Yang, M., Zhan, Y., Zhang, S., Wang, W., & Yan, L. (2020). Biological materials formed by *Acidithiobacillus ferrooxidans* and their potential applications. 3 Biotech, 10, 1-9.

Ibáñez, A., Garrido-Chamorro, S., Coque, J. J., & Barreiro, C. (2023). From Genes to Bioleaching: Unraveling Sulfur Metabolism in Acidithiobacillus Genus. Genes, 14(9), 1772.

Im, J., Lee, J., & Löffler, F. E. (2013). Interference of ferric ions with ferrous iron quantification using the ferrozine assay. *Journal of microbiological methods*, *95*(3), 366-367.

Feng, X., Liu, Q., Wang, S., Cen, L., & Li, H. (2021). Arsenopyrite weathering in acid rain: Arsenic transfer and environmental implications. *Journal of Hazardous Materials*, *420*, 126612.

Garitta, J. A., Fialho, L. L., Oliveira, G. S. D., Maria, R. M., Pirola, C., Ferreira, A. G., & Nóbrega, J. A. (2021). Microwave-assisted acid digestion: evaluation of reaction vessel design and performance. *Journal of the Brazilian Chemical Society*, *32*, 702-711.

Levine, M. (March 17, 2021) ICP-OES–ICP Chemistry, ICP-OES Analysis, Strengths and Limitations Article

Mishra, D., Kim, D. J., Ahn, J. G., & Rhee, Y. H. (2005). Bioleaching: a microbial process of metal recovery; a review. Metals and Materials International, 11, 249-256.

Rendón-Castrillón, L., Ramírez-Carmona, M., Ocampo-López, C., & Gómez-Arroyave, L. (2023). Bioleaching techniques for sustainable recovery of metals from solid matrices. Sustainability, 15(13), 10222.

Badlaa, C., & Wewers, F. (2020). Optimization of X-ray fluorescence calibration through the introduction of synthetic standards for the determination of mineral sands oxides. South African Journal of Chemistry, 73(1), 92-102.

APPENDIX A

Figure A.1: the original colour of sample ore before the bioleaching

Figure A.2: the ore sample turns grey yellow colour after bioleaching for 20 days

Figure A.3: Thermo Scientific GENESYS 20 Spectrophotometer

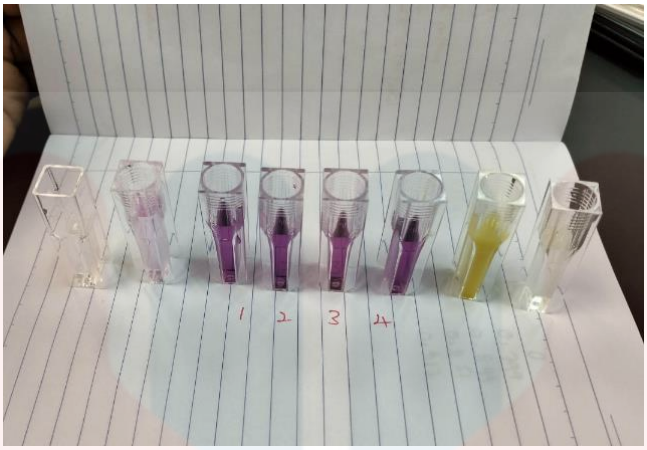

Figure A.4: the result of the ferrozine assay reaction with distil water as blank and bacterial growth culture

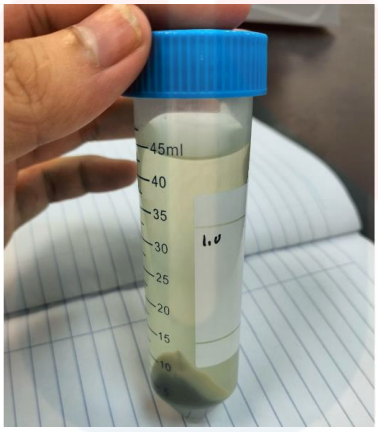

Figure A.5: sample ore run though solid liquid separation process

Figure A.6: culture medium maintains at 2.09 pH value after bioleaching

Figure A.7: Sample drying in the over

Figure A.8: Microwave digestion machine

APPENDIX B

Table B.1: Analyzation of Fe (III) oxidation activity via Ferrozine assay for 20 days

First day		
Sample	Calculation	Answer
Control	(0.038-0.016) / 0.199	0.111
0.5	(0.04-0.016) / 0.199	0.121
1.0	(0.039-0.016) / 0.199	0.116
1.5	(0.044-0.016) / 0.199	0.141
2.0	(0.046-0.016) / 0.199	0.151
4^{th} day		
Sample	Calculation	Answer
Control	(0.043-0.016) / 0.199	0.135
0.5	(0.132-0.016) / 0.199	0.583
1.0	(0.117-0.016) / 0.199	0.508
1.5	(0.146-0.016) / 0.199	0.653
2.0	(0.193-0.016) / 0.199	0.889
8^{th} day		
Sample	Calculation	Answer
Control	(0.048 -0.016) / 0.199	0.160
0.5	(0.187-0.016) / 0.199	0.859
1.0	(0.192-0.016) / 0.199	0.884
1.5	(0.227-0.016) / 0.199	1.06
2.0	(0.246-0.016) / 0.199	1.156
12^{th} day		
Sample	Calculation	Answer
Control	(0.067-0.016) / 0.199	0.256
0.5	(0.396-0.016) / 0.199	1.91
1.0	(0.432-0.016) / 0.199	2.09
1.5	(0.546-0.016) / 0.199	2.66
2.0	(0.612-0.016) / 0.199	2.995
16^{th} day		
Sample	Calculation	Answer
Control	(0.074-0.016) / 0.199	0.291
0.5	(0.648-0.016) / 0.199	3.176
1.0	(0.654-0.016) / 0.199	3.206
1.5	(0.606-0.016) / 0.199	2.965
2.0	(0.794-0.016) / 0.199	3.91
20^{th} day		
Sample	Calculation	Answer
Control	(0.081-0.016) / 0.199	0.326
0.5	(0.799-0.016) / 0.199	3.935
1.0	(0.849-0.016) / 0.199	4.186
1.5	(0.851-0.016) / 0.199	4.196
2.0	(0.913-0.016) / 0.199	4.508

Table B.2: Calculation for percentage recovery of gold from the refractory gold ore

Sample size	Au (ppm)	Calculation for percentage of refractory god	Percentage (%)
0.5g	2.112	$(2.112 - 2.009) \div 2.009 \times 100$	5.13
1.0g	2.138	$(2.138 - 2.009) \div 2.009 \times 100$	6.42
1.5g	2.168	$(2.168 - 2.009) \div 2.009 \times 100$	7.91
2.0g	2.231	$(2.231 - 2.009) \div 2.009 \times 100$	11.05

Table B.3: The data about influence of Fe removal on Au concentration following the bioleaching process

Sample Size ppm	0.5g	1.0g	1.5g	2.0g
Fe (ppm)	2.112	2.138	2.168	2.231
Au (ppm)	2.34	1.74	1.81	1.58