FENTON'S OXIDATION OF SELECTIVE HERBICIDES IN WATER USING LATERITIC IRON EXTRACTED BY *ACIDITHIOBACILLUS FERROOXIDANS* BMSNITK17

Thesis

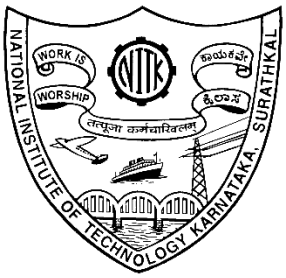
Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

BHASKAR S

(165083 CV16F06)



DEPARTMENT OF CIVIL ENGINEERING

NATIONAL INSTITUTE OF TECHNOLOGY KARNATAKA,

SURATHKAL, MANGALORE-575025

FEBRUARY, 2020

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Under the guidance of

Dr. BASAVARAJU MANU

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FEBRUARY, 2020

D E C L A R A T I O N

by the Ph.D. Research Scholar

I hereby declare that the Research Thesis entitled **"Fenton's Oxidation of Selective Herbicides in Water using Lateritic Iron extracted by *Acidithiobacillus ferrooxidans* BMSNITK17"** which is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy** in **Civil Engineering** is a *bonafide report of the research work carried out by me*. The material contained in this Research Thesis has not been submitted to any University or Institution for the award of any degree.

Place: NITK, SURATHKAL

Date:17th February, 2020

BHASKAR S
165083 CV16F06
Department of Civil Engineering

C E R T I F I C A T E

This is to certify that the Research Thesis entitled **"Fenton's Oxidation of Selective Herbicides in Water using Lateritic Iron extracted by *Acidithiobacillus ferrooxidans* BMSNITK17" submitted** by **BHASKAR S (165083 CV16F06)** as the record of research work carried out by him, *is accepted as Research Thesis* in partial fulfilment of the requirements for the award of degree of **Doctor of Philosophy**.

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BHASKAR S

Dedication

To

Dedication Sweet is his melody, Sweet is his coronet, his river with the waves
Sweet is water with lotuses galore. Sweet are all about you
O, Lord of sweetness;

Lord Krishna;

There is no shade like a mother, no resort like a mother,
No security like a mother, no other ever-giving fountain of Life.

My Mother;

Shining like a precious stone adorning the crown of a king
They stand out like a beautiful damsel in a river infested with crocodiles
They raise the devotees to the state of sovereign emperors
To such sandals I humbly offer my obeisance.

MY GURU

Dr. K S Babu Narayan;

Within the characters for thanks and feelings are embedded the symbols
for heart and speech. From the heart, with feeling, I express my gratitude.

MY SUPERVISOR

Dr. Basavaraju Manu;

With your kindness you get my attention; planting a seed of
Curiosity and Motivation on the way of my success

MY MOTIVATOR

Dr. Sreenivasa M Y

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ABSTRACT

Need for the effective, economical and eco-friendly treatment to degrade the persistent organic pollutants (POP's) is essential in day to day life. Fenton's oxidation is one of the proven technologies which have its vast application in the treatment of wide range of organic pollutants. Iron being a catalyst plays a key role demarcating its compulsion in the process. Use of commercial iron in this regard increases treatment cost. Many researchers have been carried out to replace commercial iron with natural laterite iron. Extraction of natural laterite iron by any chemical method again limits its application as its extraction adds up to the cost. Aiming at the replacement of catalytic iron in the Fenton's oxidation process a detailed study of bioleaching of iron from laterite soil was carried out and the investigation of catalytic role of extracted laterite iron in the Fenton's oxidation of selective herbicides was done. Novel bacterial strain was isolated and characterized at molecular level by gene sequencing technique and the sequence was submitted to Genbank to get an accession number. Isolated bacteria confirm to be an acidophilic chemolithotrophic bacterium *Acidithiobacillus ferrooxidans* belongs to the gamma proteo bacterial group with an accession number MG271840. Iron mineral biologically synthesized using isolated strain *Acidithiobacillus ferrooxidans* BMSNITK17 was characterized and confirms to be biogenic jarosite with XRD and EDS technique. This iron mineral was evaluated for its catalytic role in Fenton's oxidation for the degradation of ametryn and dicamba. The fresh biogenic jarosite in Fenton's oxidation was found to degrade ametryn by 84.90 % following alkylic oxidation and hydroxylation pathway which was confirmed with mass spectroscopy studies. Whereas the same mineral shows 91.29 % of dicamba degradation with Fenton's oxidation process promising cost effective treatment.

System conditions like pH, feed mineral particle size, pulp density, temperature, rotor speed has an effect on bioleaching potential of *Acidithiobcillus ferrooxidans* BMSNITK17 in leaching out iron from laterite soil. Very fast iron dissolution was observed with laterite and soon the drop in the iron concentration of leached solution. The drop in total iron concentration was due to the precipitation of leached iron. The

leaching conditions were optimized in the current study with respect to the native bacterial strain *Acidithiobacillus ferrooxidans* BMSNITK17. Maximum iron concentration leached out accounts to 281.0 mg/L under system conditions like pH 3.0, temperature 30 ^{o}C, pulp density 5%, shake flask speed 180 RPM and particle size 150 µm. The bioleached laterite iron (BLFe's) on evaluation for its catalytic role in Fenton's oxidation for the degradation of ametryn and dicamba exhibits 94.24 % of ametryn degradation and 92.45% of dicamba degradation efficiency. Fenton's oxidation performed well with the acidic pH 3. The process follows pseudo first order reaction.

Our findings suggest the application of biogenic iron mineral jarosite and bioleached laterite iron as a catalyst in the Fenton's Oxidation process for treating hazardous herbicides which are the part of an agricultural runoff. The study marks the low cost treatment of hazardous pollutants using naturally available minerals.

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NOMENCLATURE

AOP	:	Advanced Oxidation Process
AMD	:	Acid Mine Drainage
BLFe	:	Bioleached Laterite Iron
COD	:	Chemical oxygen demand
BOD	:	Biochemical oxygen demand
POP	:	Persistant Organic Pollutants
DNA	:	Deoxyribonucleic Acid
REDOX	:	Reduction and Oxidation Potential
XRD	:	X – Ray Diffraction
SEM	:	Scanning Electron Microscopy
EDS	:	Energy Dispersive X- Ray Spectroscopy
16S rRNA	:	16S ribosomal Ribonucleic Acid
ATP	:	Adenosine Triphosphate
NAD /	:	Nicotinamide adenine dinucleotide
NADH	:	Proton Motive Force
PMF	:	Acid Mine Drainage Collection Tank
AMD CT	:	Polymerase Chain Reaction
PCR	:	Basic Local Alignment Search Tool
BLAST	:	National Center for Biotechnology
NCBI	:	Information
ESI–MS	:	Electrospray Ionization Mass Spectrometry
BET	:	Brunauer – Emmett – Teller
RPM	:	Rotation per minute
PD	:	Pulp density
ATCC	:	American Type Culture Collection
PDF	:	Powder Diffraction File
Eqn	:	Equation

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CHAPTER 1

INTRODUCTION

1.1 GENERAL

Evolutionary movements in civilization and industrialization over decades have put us in an alarming zone. Shift from organic farm to chemically monitored agricultural method to increase the crop yield by means of herbicides and pesticides application to control weeds and pests, in one another way degrades environmental quality. Engineers and technocrats in this concern are on road to find the remedial measures and also to prevent and control the possible hazards. Worldwide application of herbicide to increase the significant yield of agricultural production by controlling weeds and pests may in turn have adverse effect with environmental pollution. It is the bioaccumulation, lipophilic property, long half-life and large range transport that make herbicide more persistent in the environment. Persistent Organic Pesticides (POP's) are the class of herbicides which includes organochlorides that pose hazard to the environment. Pesticides like aldrin, isobenzane, pentachloro phenol are likely to be classified as highly hazardous exposure to which pose neurotoxicity and DNA damage. Most of the herbicides are likely to present for a longer period deteriorating environmental quality (Grant 1979; Gupta 2017; Jayaraj et al. 2016). Commercial agricultural crops like sugarcane, paddy and popcorn are generally prone to effected by undesirable growth of broad leaf and weeds. Ametryn, a derivative of S- triazine is widely recommended as an effective herbicide against such weeds in agricultural practices (Lopez et al. 1997). The peculation and movement of this compound to water bodies from the surface of the soil prevails a toxic environment to the aquatic ecosystem including (Gaggi 1995). Dicamba is another class of benzoic acid herbicide formulated to substitute atrazine compounds in keeping woody plants and broad leaf woods (Gupta 2017; Junghans et al. 2006; Lebaron et al. 1952). Dicamba reaches surface and ground water thereby contaminating the water source. Surface runoff and leaching contributes to maximum herbicide contamination. Movement and persistence of this herbicide in the

environment depends on many factors which makes it difficult to predict (Burnside et al. 2008).

Advanced oxidation process is one of the promising technologies in the degradation of hazardous herbicides among which Fenton's oxidation proves to be much effective (Chen et al. 2016). Use of H_2O_2 for the degradation of triazine compounds has been proved efficient by many researchers. Many researchers have shown that Fenton's oxidation process is effective in the degradation of organic pollutants (Brillas et al. 2003; Chamarro et al. 2001; Tantak and Chaudhari 2006). Iron playing catalytic role in the production of hydroxyl radicals which attacks the target compound increase the efficiency of process reducing the treatment time. Larson and co-workers (1991) investigated the catalytic action of ferric in the photooxidation of triazine compounds claiming the use of ferric enhances the degradation by two to three orders of magnitude. Use of iron as a catalyst either in ferric or ferrous form as a catalyst in Fenton's oxidation adds up to the treatment in large quantity (Larson et al. 1991). Industrial application of Fenton's treatment at large scale fails with the investment to commercial iron and hydrogen peroxide. To overcome this research has been done to replace commercial iron with laterite iron extract by chemical processing (Karale et al. 2013; Manu, B and Amritha A. 2018; Sangami and Manu 2017, 2018). Chemical extraction of iron from laterite soil being expensive fails at large scale application.

Application of Microorganisms as a bio degradation tool in pesticide degradation is recommended as safe and eco-friendly with that of physio chemical mechanisms. Scientists have successfully endeavored the tool of application of microorganisms in recovering the valuable minerals indifferent and substandard ores which lead us to new technology called 'Bio mining' or 'Bioleaching'. The term Bioleaching refers to the metamorphosis of the naturally available ore to soluble state. The oxidative reactions of bioleaching when mediated by the microorganisms are called Biooxidation. Among the wide variety of microorganisms in leaching out minerals from an ore, a group of proteobacteria, archea found to be most effective and metal tolerant. These are acidophilic organisms does exist in acid mine drainage of mining sites (Baker and Ban 2003). *Acidithiobacillus ferrooxidans*, chemolithotrophic acidophilic gram negative

bacteria which relay on iron oxidation for its energy source and can reduce sulphur is extensively used in the bioleaching process (Acevedo and Gentina 1989). Studies have been carried out to evaluate the efficiency of these organisms in leaching and models have been proposed to describe its mechanism. *Acidithiobacillus ferrooxidans* is an acidophilic bacterium which is studied extensively at molecular level so far. The other acidophilic thermophiles, moderate thermophiles are still under study.

Bioleaching has a long history. Unknowingly the early miners were using this technique. Recovery of copper from local deposit of Seville in south spain from pre-romans was the first historical bioleaching episode that has been reported. Later on this is come to be known as Rio Tinto Mine. Red color of water due to high ferric concentration imparted by microbial oxidation gives the river name 'Rio Tinto Mine' (Gómez et al. 2002). Brandl (2001) carried out a pilot scale studies of Bioleaching of copper and zinc with a mixed bacterial consortium of *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans* and *Leptospirrillum ferrooxidans* and found that maximum leaching can be obtained with mixed population of bacteria with a temperature of 30^{o}C and 5% pulp density with iron being precipitated as jarosite (Brandl 2001). Eisapour and coworkers (2013) studied the process of bioleaching in a batch stirred tank reactor for uranium extraction by pure isolates of *Acidithiobacillus ferrooxidans* and found 95% of efficiency (Eisapour et al. 2013). Wu and his team. (2016) performed column leaching studies on low grade secondary copper sulphide ore with native AMD microorganisms and found copper leaching of 90% in 240days (Wu et al. 2016). Latorre et al. (2016) studied the bacterial consortium with five different isolates and trace out the role of each species in the consortium by genomic data analysis and found its leaching efficiency better than a single isolate (Latorre et al. 2016). The attempt has been made to use fungus in leaching minerals from ore. The use of fungus in bioleaching has been studied so far and it has not focused much as the efficiency is comparatively less and also because of chance of contamination. Sukla et al., (1995), studied the efficiency of fungal strain *Aspergilus Niger* in leaching copper, cobalt and Nickel and found the fungus is more efficient in recovering cobalt (50%) from converter slag and less efficient in recovering nickel (23%) at 37^{o}C of temperature within 72 hours (Sukla et al. 1995).

The present study the attempts to isolate iron oxidizing bacteria and characterizing the isolated strain by molecular techniques. On screening by iron oxidation activity, the dominant strain is used to evaluate for its bioleaching potential in leaching out the iron from the lateritic soil under optimized conditions. The catalytic activity of the leached iron in Fenton's reaction to degrade the selective herbicides.

1.2 OBJECTIVES

The objective of the current research is bioleaching of Iron from laterite soil using iron oxidizing bacteria.

Specific objectives

1. Isolation and molecular characterization of iron oxidizing bacteria from Acid Mine Drainage (AMD). Also to synthesize iron mineral using isolated strain.
2. To investigate the bioleaching potential of isolated strain for iron leaching from the laterite soil.
3. To assess the utility and catalytic activity of biosynthesized iron mineral and leached iron as Fenton's reagent for the degradation of dicamba and ametryn in aqueous solution.

1.3 MOTIVATION FOR THE RESEARCH

In spite of the extensive study on the bioleaching by various researches the process provides a wide scope for the more studies in relation to the organism involved in the process and mechanism of leaching. The interdependent characteristic feature of key organism involved in the process, optimal conditions for leaching, type of metal to be recovered from the parent ore and the structure of ore made research scientists to have intensive look into the process. Two distinguish areas the process involved is microbiology dealing with the leaching agents and the process engineering in relation with the leaching mechanism. The current study focuses these two areas in perspective to the leaching of iron from the laterite soil available in local. On the hand, out of the environmental concern to herbicide contamination by the agricultural runoff and herbicide manufacturing industry the need for its removal is becoming major problem. Advanced oxidation process is advised for the treatment of organic pollutants for its

efficiency and short duration of treatment among which Fenton's oxidation is proven to be more suitable. The need for cost effective treatment arise with Fenton's treatment as the process demands catalyst. Replacement for the commercial iron arises with the replacement suggesting natural iron. Bioleaching extensively used for the low cost metal recovery technique has a potential to extract natural iron from laterite soil which could be advised as a replacement for the commercial Fenton's catalyst.

The present research has been undertaken by considering the following points indicating the research gap.

- The dearth for a research on the bioleaching of iron from laterite ore bearing high content of iron, extraction of which is not cost effective and environmental friendly by the physical and chemical method.
- The ambivalence in understanding the interaction between the ore body and microbiology biology of iron oxidizing bacteria at its root.
- The assessment of catalytic activity of type of iron leached from biooxidation on the Fenton's oxidation process.

Previous studies emphasize the mechanism of bioleaching and factor affecting the processes which are the key drivers in recovering valuable minerals from ore and seem to be economical from the marketing point of view. There have been various attempts by researchers to evaluate the efficiency of leaching organisms in recovery by optimizing the various limiting factors. Bio correlation between the molecular biology and biooxidation of iron has not been studied much.

Bioleaching of copper, arsenic, gold, chromium has been studied so far using different bacterial strains like *Acidithiobacillus ferrooxidans*, *Leptosprillum ferrooxidans*, *Acidithiobacillus thiooxidans*, *Ferroplasma* etc, Bioleaching experiments have been studied using fungal strains like *Aspergilus Niger*, *Pencillium verruculosm* for iron leaching but bioleaching of iron from laterite soil employing bacteria has not been studied so far. Study on the Fenton's degradation of dicamba was studied (Brillas et al. 2003). Effect of various controlling factor on catalytic activity of biosynthesized iron particles in Fenton's degradation of phenol was studied and interpreted by yan and co – workers (Yan et al. 2017). The present study focuses on the isolation and molecular

characterization of iron oxidizing bacterial strain and employing the isolated bacterial strain in recovering the iron present in the laterite soil. The iron extracted will be used as a catalyst for Fenton's degradation of dicamba and ametryn.

1.4 STRUCTURE OF THE THESIS

This section describes the main elements of thesis written,

Chapter 1 of the thesis introduces the reader to the present study with brief and conceptualized note. This chapter covers the means of research, motivation, research gap along with the objectives.

Chapter 2 provides one the brief literature survey carried out which deals with the study of process and mechanism of bioleaching with insight to microbial biooxidation, microorganisms involved in the process of bioleaching, Selective herbicides and its fate in the environment and Fenton's oxidation, an effective tool in the degradation of organic pollutants. This encompasses the research that has been already carried out in relevant to the present study and brief research outcome of the previous study. The chapter highlights the need for current study.

Chapter 3 gives the plan and methodology adopted to accomplish the objectives framed. Chemicals, reagents used for experimental investigation is addressed with the grade. The methods are explained in detail. Standard experimental procedure followed for the analysis is given in detail in the Annexure part at the end of the thesis.

Chapter 4 presents before the outcome of the research in detail. Experimental and graphical representation of the analyzed data is presented in this section along with the discussion in detail. Consistency of the result obtained in the present work to that of previous research and its relevancy has been presented.

Chapter 5 concludes the research objectives based on the obtained results and interpretations. This section concludes the research accomplishments and contribution.

CHAPTER 2

LITERATURE REVIEW

2.1 BIOLEACHING: A BIOHYDROMETALURGICAL APPPROACH

Bioleaching is the process of biological oxidation of almost insoluble ores through the mobilization of metal cat ions. The technology is widely applicable for the extraction of metals such as copper, cobalt, nickel, zinc and uranium (Vera et al. 2013). The biooxidation of insoluble metals are catalyzed by the acidophilic microorganisms. These organisms are now a day's employed for the recovery of metals from the ores in industry referred to as Biomining. The use of naturally occurring key components like microorganisms, water air, at ambient conditions has taken this technology from laboratory to industry. Microbe based process has its economic advantage in the extraction of metals from low grade deposits. Low grade mineral ores which cannot be processed with normal mine processing has been subjected to bioleaching for the recovery of copper. This also includes the dumps left over in mining operations of which valuable metals can be recovered by means of insitu heap leaching process. Bioleaching operation is most preferable as it is ecofriendly in nature and does not involve the consumption of large amounts of energy as in roasting, smelting and do not produce sulphur di oxide and other harmful gaseous emissions.

Bioleaching of iron rich sulphide minerals is carried out by acidophilic microorganisms such as *Acidithiobacillus ferrooxidans, Leptospirrilum ferrooxidans, Sulfolobus* and so on in an acidic medium (Watling 2006). Commercial Bioleaching methods are insitu, dump, heap and vat leaching. The technique of insitu leaching involves pumping of a liquid solution with a mixture of air under compressed force deep into a mine surface or even directly to the ore surface which is made pervious by explosion. The resulting refined liquids are retrieved through specially designed wells drilled under the ore remains. In dump leaching uncrushed waste rock which has low metal content to recover by conventional procedure is piled up and the lixiviant solution is poured onto it which seeps to the bottom by the gravity flow on the other hand heap leaching involves the preparation of ore primarily by size reduction to promote mineral lixiviant

interaction. In both the methods the dilute sulphuric acid is sprinkled on top allowing it to percolates down the dump lowering the pH which in turn promotes the growth of acidophilic microorganisms. Acid runoff is collected at the bottom and the metal is extracted by the solvent extraction or electro winning process. Vat leaching is the controlled bioleaching operation usually carried out in bioreactors which brings out the controlled operation. However this technology involves the higher cost (Siddiqui M. H. et al., 2009). Biohydrometallurgy, an interdisciplinary field that includes various aspects of hydrometallurgy, mineralogy, geo-microbiology, chemical and mining engineering is considered to be one such broad area deals with the bioleaching of metals from low grade ores.

2.2 MICROORGANISMS IN BIOLEACHING PROCESS

The dominant microorganisms capable of oxidizing the iron and sulphur compounds are of key interest to the researchers because of its unique nature in tolerance to the high metal concentration and acidic pH. Studies conducted on the involvement of bacteria, fungi, yeasts, algae and protozoa reveals that there is a wide scope in employing these bacteria to recover valuable metals. Out of these bacteria proves to be more efficient in leaching metals from low grade ores. The most studied iron and sulphur oxidizing acidophilic bacteria is *Thiobacillus ferrooxidans*. Now it is called as '*Acidithiobacillus ferrooxidans*' once it is renamed by Kelly and wood in 2000. Prevailing chemolithotrophic mode of mechanisms of number of acidophilic bacteria supports their growth on low pH environment as the abundance of inorganic sources are more compare to organic source at low pH conditions. Among the bacteria most studied group of bacteria are ɣ-Proteobacteria *Acidithiobacillus ferrooxidans*, a dominant iron – oxidizing bacteria in acid mine drainage habitat. Another group of bacteria belongs to archea *Thermoplasmatales* and the *Sulfolobales* are studied so far but genomics, proteomics are still under research. Culture-independent methods have provided a detailed understanding of the full diversity and phylogeny of organisms populating AMD systems. Genome sequences of few isolates like *F. Acidarmanus* and *A. ferrooxidans* has been known till date that would made researchers to understand the biochemistry of acidophilic organisms that deals with the bioleaching. This knowledge

has been limited to few microbes excluding a wide variety of acidophiles which has not been isolated yet (Baker and Ban 2003).

2.2.1 Mesophiles

Acidithiobacillus ferrooxidans

This is a Chemolithotrophic aerobic rod shaped bacteria, cells ranging in size by 0.3 to 0.8 micrometers in diameter and from 0.9 to 2 micrometer in length (Barreto et al. 2003). The bacteria is extremophile with a predominant acidophilic property which makes it to grow in highly acidic pH from 1.5 to 4. The bacterial growth and iron oxidation occurs at a temperature of 20 ^{o}C to 40 ^{o}C (M Nemati and Harrison S.T.L 2000). Since the bacteria exhibits autotrophic aerobic growth it derives its carbon dioxide as a cellular carbon source and oxygen as electron acceptor from the ambient air. The oxidation of ferrous ion to ferric ions and oxidation of reduced sulphur compounds occur in the bacteria cell made them an interest of several researchers. Assimilation of carbon occurs via Calvin-Benson cycle in the form of carbon-dioxide. Under anaerobic conditions bacteria grows on elemental sulfur or metal sulfides using ferric ions as electron acceptor. Hence it is classified as obligate aerobes (Brandl 2001; Cerruti et al. 1998). *Acidithiobacillus ferrooxidans* is not favored in situations in which the ferric content is much higher than ferrous iron i.e high redox potential (Rawlings and Johnson 2007). Phylogenetic studies indicates the genus *Acidithiobacillus* is situated close to the branch point between β- and ɣ - subdivisions of the proteobacteria (Coram et al. 2002).

Leptospirrilum Ferrooxidans

The highly acidic gram negative, chemolithotrophic bacteria grows in a pH range from 1.4 - 1.8. On phylogeny these are the members of the division Nitrospira. The capability of using only ferrous ion as electron donor differentiated this genus from others and made them unique in nature. *Leptospirrilum ferrooxidans* alone are widely used in biooxidation because of its high capability to oxidize iron and sometimes in combination with sulphur-oxidizing bacterium like *Acidithiobacillus caldus* and *Acidithiobacillus thiooxidans*. Hippe (2000), isolated and identified two species belong to leptospirrilum genus, *Leptospirriulum ferrooxidans* and *Leptospirrilum*

thermoferrooxidans based on 16S rRNA technique. *Leptospirrilum thermoferrooxidans* were reported to grow at 45 ^{o}C but does not exhibits growth as other acdophiles. Four species of leptospirrilum exist till date. *Leptospirrillum ferrooxidans* belongs to two GCC groups 49 %-51 % and 55 %-56 % (Coram et al. 2002). Since two species of *Leptospirrilum ferrooxidans* belongs to GCC groups less than 49 %-51 %, higher GCC group is recognized as third species, named *Leptospirrilun ferriphilum* (Coram et al. 2002). It is important to note that the existence of a fourth species is suggested by 16S rRNA sequence data from the total DNA extracted from abandoned pyrite mine samples but no members of this proposed fourth species have been isolated in laboratory (Bond et al. 2000).

2.2.2 Moderate Thermophiles

The precise temperature does not exists to divide mesophilic activity from moderate thermophilic activity. Activity of *Acidithiobacillus caldus* and *Acidimicrobium* are seen in the temperature range of 25 ^{o}C to 55 ^{o}C. Generally mesophilic bacteria shows their optimum activity below 40 ^{o}C and moderate thermophilic bacteria have an optimum temperature of above 45 ^{o}C. The sulfobacillus genus was well known for a moderate thermophiles, *Sulfobacillus thermosulfidooxidans* one. Genus Sulfobacilli are gram positive, endospore forming bacteria isolated from heaps of mineral waste. Some of the bacteria under this genus are heterotrophic in nature. On heterotrophic mode of metabolism glucose is the carbon and energy source for the bacteria. These bacteria are also exhibits their growth in the absence of oxygen being an obligate heterotrophs. Under such circumstences it is the ferric ions serves as final electron acceptor and sulphur compounds the final electron donors (Johnson and Hallberg 2009). *Acidimicrobium ferrooxidans* is another moderate thermophile widespreaded in acidic natural geothermal sites and mineral sulphide mine environments (Clark and Norris 1996). *Ferroplasma acidiphilum* an microbe which are likely to be archea. This bacteria was isolated first from the arsenopyrite treating pilot plant bioreactor and studied in detail (Golyshina et al. 2019). *Ferroplasma acidarmanus* was another closely related archeaon isolated from acid mine drainage (Edwards et al. 2000; Golyshina et al. 2019).

2.2.3 Thermophiles

These organisms are actively grown at high temperature with autotrophic or heterotrophic mode of growth. The lowest optimum temperature among the thermophiles studied so far is about 68 ^{o}C i.e for *Sulfolobus metallicus* (Clark and Norris 1996). Sulfolobus strain is the most dominant one in most of the studies with a operating temperature of 65 ^{o}C – 70 ^{o}C for mineral sulphide oxidation but most species were not identified in specific (Gericke et al. 2001). It has been noted that *Metallosphaera sedula* and various unnamed isolates carry out the mineral oxidation at 75 ^{o}C. Novel isolates capable of growing on pyrite and chalcopyrite has been observed at a temperature of 88 ^{o}C and 90 ^{o}C and copper extraction was observed in maximum at 85^{o}C (Norris and Owen 1993; Plumb 2002).

2.3 MECHANISM OF BIOLEACHING

The model for mechanism of bioleaching was first proposed by Silverman and Lundgren in 1959. In case of direct leaching it is the bacterial membrane which interacts directly with the sulfide by means of enzymatic action. Hence this type of leaching is seen only if the cells attach to the mineral surface (Brandl 2001). *Acidithiobacillus ferrooxidans* and *Leptospirrilum ferrooxidans* have strong affinity for mineral surfaces to which they rapidly attach (Devasia et al. 1993; Sand et al. 1995). The Extrapolysaccharide (EPS) layer produced by the bacteria attaches it to a mineral surface and forms the matrix in which the cells divide and eventually form a biofilm (Tributsch 2001). The mechanism of microbial oxidation is carried out by the oxidation of ferrous ions which is available in the mineral ore in case of indirect leaching (Brandl 2001) . The ferric ion being an oxidizing agent oxidizes metal sulphides which are further chemically reduced to ferrous ions. In this case the no straight or bodily association is required for oxidation of iron. Ferric ion being an oxidizing agent oxidizes metal sulphide and is chemically reduced to ferrous ions. Here no direct physical contact is needed for the oxidation of iron.

Direct Mechanism

$$MS + 2O_2 \xrightarrow{\text{T. Ferrooxidans}} M_2+ + SO_4^{2-} \quad \text{(Eqn i)}$$

In this action the metal sulphides are directly oxidized to a fine soluble metal and its sulphates by *Acidithiobacillus ferrooxidans.* Generally the available metal sulphides exist in solid insoluble deposition where the metal sulphates are water soluble in nature. This reaction transforms a insoluble solid phase to a soluble liquid state which can be recovered to a metal state by further treatment. The mechanism can be continued until all the substrate (MS) is converted to product (MSO_4)

Pyrite: $4FeS_2 + 15O_2 + 2H_2O \rightarrow 2Fe_2 (SO_4)_3 + 2H_2SO_4$ (Eqn ii)

Chalcopyrite: $4CuFeS_2 + 17O_2 + 2H_2SO_4 \rightarrow 4CuSO_4 + 2Fe_2 (SO_4)_3 + 2H_2O$ (Eqn iii)

Chalcocite: $2Cu_2S + 5O_2 + 2H_2SO_4 \rightarrow 4CuSO_4 + 2H_2O$ (Eqn iv)

Covellite: $CuS + 2O_2 \rightarrow CuSO_4$ (Eqn v)

Sphalerite: $ZnS + 2O_2 \rightarrow ZnSO_4$ (Eqn vi)

Indirect mechanism

In this mechanism, bacteria does not come in contact with sulphide minerals instead the oxidation of same occurs by ferric ions. Bacterial oxidation of ferrous ions to ferric ions plays an important role here.

$$4FeSO_4 + 2H_2SO_4 + O_2 \xrightarrow{\textit{Thiobacillus ferrooxidans}} 2Fe_2(SO4)_3 + 2H_2C \quad \text{(Eqn vii)}$$

The oxidation elemental sulphur also occurs by bacteria.

$$2S^o + 3O_2 + 2H_2O \xrightarrow{\textit{Thiobacillus ferrooxidans}} 4H^+ + 2SO_4^- \quad \text{(Eqn viii)}$$

Metal dissolution occurs by a cyclic process and the formation of H^+ during the sulphur oxidation enhances the overall reaction efficiency. Model that has been developed so far including hypothetical theories to explain direct and indirect bioleaching mechanism is still under discussion (Nemati et al. 1998).

Though there is no evidence available for direct enzymatic leaching, two indirect mechanism have been proposed to explain the bioleaching process (Sand et al. 1995, 1999; Sand and Gehrke 2006). Sand and coworkers observed the oxidation of different metal sulphides occurs through intermediates like thiosulfate and polysulfate and hence by concluded the dissolution is not identical for all metal sulpfides. The two models have been proposed based on the intermediates formed during the dissolution reaction, i.e thiosulfate mechanism and polysulfate mechanism.

In Thiosulfate mechanism, thiosulfate being main intermediate and sulfate being end product, the solubilization of acid insoluble metal sulfide occurs through ferric iron attack on the ore (Sand et al. 1995).

$$FeS_2 + 6Fe^{3+} + 3H_2O \longrightarrow S_2O_3^{2-} + 7Fe^{2+} + 6H^+ \qquad \text{(Eqn ix)}$$

$$S_2O_3^{2-} + 8Fe^{3+} + 5H_2O \longrightarrow 2SO_4^{2-} + 8Fe^{2+} + 10H^+ \qquad \text{(Eqn x)}$$

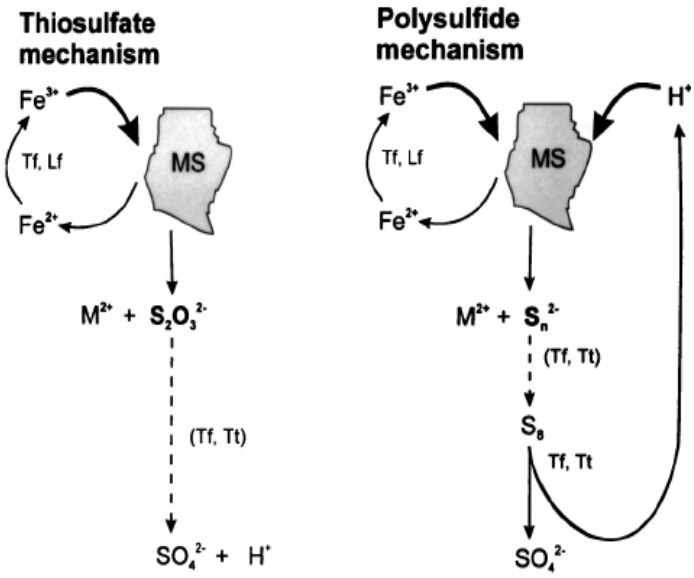

Figure 2. 01. Mechanism of Bioleaching (Vera et al. 2013)

In polysulfide mechanism, the solubilisation of acid soluble metal sulphide occurs through combination of ferric ions and protons with elemental sulphur as the main intermediate. This elemental sulphur is relatively stable but may be oxidized to sulphate by sulphur oxidizing bacteria. Microorganism's plays a major role in producing sulfuric acid for proton hydrolysis attack during the metal solubilization process and helps to keep iron in oxidative ferric state for an oxidation attack on the mineral.

$$MS + Fe^{3+} + H^{+} \longrightarrow M^{2+} + 0.5\ H_2S_n + Fe^{2+} \quad \text{(Eqn xi)}$$

$$0.5\ H_2S_n + Fe^{3+} \longrightarrow 0.125\ S_8 + Fe^{2+} + H^{+} \quad \text{(Eqn xii)}$$

$$0.125\ S_8 + 1.5O_2 + H_2O \longrightarrow SO_4^{2-} + 2H^{+} \quad \text{(Eqn xiii)}$$

Metal sulphides such as sphalerite (ZnS), galena (PbS), arsenopyrite (FeAsS), chalcopyrite ($CuFeS_2$) and hauerite (MnS_2) are ammeanable to polysulfide mechanism, in which the chemical bonds between metal and sulphur moiety can be broken by ferric ion and by protein attack.

2.4 MOLECULAR BIOLOGY OF ACIDITHIOBCILLUS FERROOXIDANS

Acidithiobacillus ferrooxidans, a gram negative gamma proteobacteria has been studied extensively by the researchers so far. In spite of availability of some of model to explain electron pathways iron oxidation metabolism, no sufficient data are available to explain sulphur oxidation. Iron and sulfur oxidation has been derived from *Acidithiobacillus ferrooxidans* strains out of which some have not been characterized phylogenetically (Quatrini et al. 2009). Quatrini et al., (2005) identified candidate genes responsible for iron uptake, sensing, storage and regulation of homeostasis (Quatrini et al. 2005). They detected difference in amino acid sequence that suggest ways in which the periplasmic and outer membrane proteins of *Acidithiobacillus ferrooxidans* maintain structural integrity and relevant protein-protien contacts at low pH. Quatrini et al. (2009) identified the involvement of some of the novel genes like cup (copper oxidase), ctaABT (heme biogenesis and insertion), nuol and nuoK (NADH complex sub units), sdrA1 (NADH complex accessory protein), atpB and atpE (ATP synthetase F0 subunits) in iron oxidation pathway. Quatrini and co-workers proposed a model which includes some specific candidate genes and putative proteins in the mechanism of sulphur assimilation. *Ferroplasma acidiphillum* belongs to a group of archea which lacks cell wall and genetically more similar to the order Thermoplasmales with 87.2% of similarity (Golyshina et al. 2019).

2.5 BIOOXIDATION OF IRON

The oxidation of ferrous iron by *Acidithiobacillus ferrooxidans* is considered to proceed in a 'downhill' and an 'uphill' reaction. In the downhill pathway, electrons removed biologically from ferrous proceed through a series of electron carriers, from the outer membrane to the cytoplasm where they are used to reduce oxygen to water consuming protons in the process. *Acidithiobacillus ferrooxidans* has to generate the reduced pyridine nucleotides NADPH and NADH necessary for CO_2 and N_2 fixation and other anabolic processes. Because of the standard reduction half-potential of the Fe(II)/Fe(III) couple is very much positive than that of NAD(P)/NAD(P)H couple, the electrons have to be pushed uphill from Fe(II) to NAD(P) against the redox potential gradient. This uphill electron transfer requires energy which is probably provided by the proton motive force (PMF) and the uphill flow of electrons can be considered somewhat similar to a mitochondrion working in reverse. In addition, ATP hydrolysis via the ATP synthetase working in reverse may be used to generate an electrochemical proton gradient that may provide some of the force to push electrons 'Uphill' (Coram et al. 2002). Holmes and Bonnefoy (2009) showed that Leptosirilum genus having G+C content 55%-58% grows at 45°C are efficient in bio oxidation at South African commercial bioleaching tank (Barton et al. 2010; Quatrini et al. 2005, 2006).

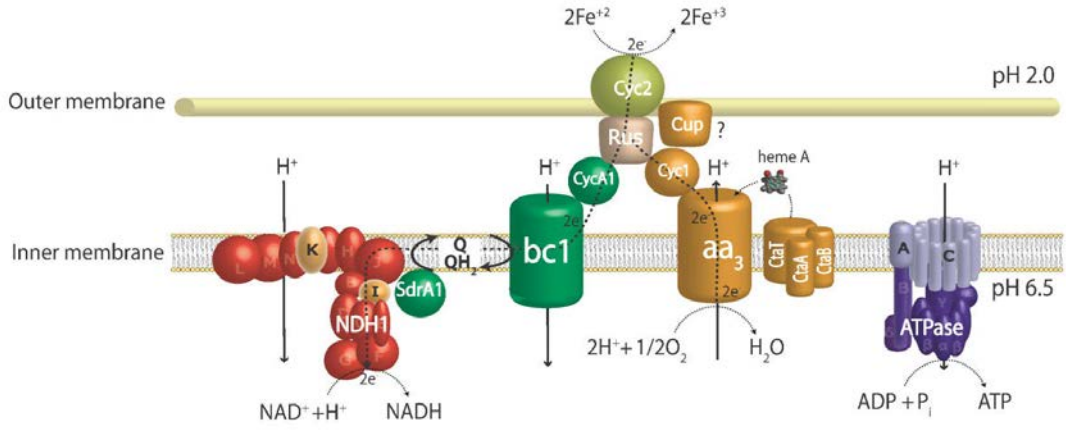

Figure 2. 02. Mechanism of Iron oxidation in *Acidothiobacillus ferrooxidans*
(Quatrini et al. 2009)

2.6 SELECTIVE HERBICIDES:

Ametryn:

Ametryn, a derivative of S- triazine is widely recommended as an effective herbicide against such weeds in agricultural practices (Lopez et al. 1997). It is reported that the excess application of ametryn results in the contamination of the ground water and in the due course the seepage of this compound leads in contamination of ground water. This has resulted as a high risk to aquatic species and unsafe for portable purposes (Kasozi et al. 2012). The peculation and movement of this compound to water bodies from the surface of the soil prevails a toxic environment to the aquatic ecosystem including (Gaggi 1995).

Dicamba:

Dicamba is a class of benzoic acid herbicide formulated to substitute atrazine compounds for the control of woody plants and broad leaf weeds (Gupta 2017; Junghans et al. 2006; Lebaron et al. 1952). Dicamba is primarily a solid and liquid as herbicide but also available in pellets and granules for commercial applications. Approximately the recommended application rate of dicamba varies in the range 0.114 kg/ha for barely to 0.294nkg/ha for corn. Characteristic feature of dicamba exhibits a low vapor pressure of 4.5 mPa at 25 ^{o}C, high water solubility of 6.5 g/L at 25 ^{o}C with a melting point between 114 ^{o}C and 116 ^{o}C (Caux, P. et al. 1993). Dicamba reaches surface and ground water thereby contaminating the water source. The herbicide is toxic to aquatic life at chronic dosage may even kill aquatic life like fish as it is water soluble with >10 % (wt/vol) application (Wauchope 1978). Volatilization and eolic erosion transfer the herbicide by air flow. However the vapor loss of applied herbicide are relatively small and also depends on the local climatic conditions (Dinelli et al. 1996). Sandmann and co-workers (1991), studied the atmospheric presence of dicamba near tala valley South Africa with an evident of 0.1 µg/L of dicamba in 500 ml of rain sample. Surface runoff and leaching contributes to maximum herbicide contamination. Dicamba is another class of benzoic acid herbicide formulated to substitute atrazine compounds for the control of woody plants and broad leaf weeds. Movement and persistence of this herbicide in the environment depends on many factors which makes it difficult to predict (Burnside et al. 2008).

2.7 FENTON'S OXIDATION

Fenton's reagent is mixture of H_2O_2 and ferrous iron, which on dissociation produces highly reactive hydroxyl radicals capable of degrading the organic pollutants. The process is discovered by H J Fenton while oxidizing polycarboxylic acids with H_2O_2. The Fenton's oxidation process, a type of advanced oxidation processes proved to be efficient in degrading most of the hazardous organic pollutants from water and waste water converting them harmless compounds like CO_2, water and inorganic salts. Successful attempt have been made by Karale et al., 2013, in replacing the laterite extracted iron to traditional iron for the removal of 3-Aminopyridine with a removal efficiency upto 82%. The study promises the use of iron extracted from the laterite soil by chemical leaching method can be used as iron catalyst in the Fenton's like reaction. Yan and co-workers (2017) made a study on Fenton's oxidation evaluating catalytic role of jarosite and schwertmannite synthesized biologically using *Acidithiobacillus ferrooxidans* for the degradation of phenol. The use of schwertmannite proved to be much more efficient in exhibiting catalytic capacity compare to jarosite in Fenton's process while the jarosite exhibited degradation of phenol in much wider pH range i.e 3.5-7.5 over schwertmannite which is more efficient in the pH range of 3.0-4.5 (Yan et al. 2017).

In addition, iron oxide magnetic nanoparticles are shown to be very effective catalyst in heterogeneous Fenton's like reaction in the degradation of organic pollutants. Chen et al., 2017 synthesized iron oxide nanoparticles by ionothermal process and proved these iron oxides exhibit highest catalytic activation energy up to $47.6 kJm^{-1}$ in the degradation of RhodamineB (RhB) (Chen et al. 2017).

Table 2. 01. Summary of Literature Survey

YEAR	EVENT	RESEARCHER	IMPACT
1947	Identification of *Acidithiobacillus ferrooxidans*	Colmer & Hinkle	Revolution in the field of Hydrometallurgy
1958	Patent for describing Ferrous Iron Oxidation by bacteria	Zimmerley et al.,	
1993	Use of Acidophilic microbes as bioleaching agents	• Bailey & Hansford • Ahonen & Tuovinen • Westbroek et al.,	Industrial Application of Microorganisms for the leaching of minerals
1998	Reactor studies on optimization of leaching condition	Gomez et al.	Optimization of Leaching Conditions
2005	Study on Formation of Ironhydroxy sulphate precipitates by *Acidithiobacillus ferrooxidans*	Daoud & Karamanev	
2006	Isolation and molecular characterization of *Acidithiobacillus ferrooxidans*	Chen et al.	• Species Specific primers • Enzyme Specific primers
2008	Study on the effect of iron type in Fenton's reaction on biodegradability of hazardous organic compounds	Khan et al.	
2012	Study on effect of ferrous ion oxidation rate on secondary ironhydroxy sulfate mineral	Huang & Zhou.	

2013	Study on Magnetic properties of iron oxidizing bacteria	Yan et al.,	Identified Magnetosomes in Iron Oxidizing Bacteria
2013	Study on catalytic activity of leached iron in Fenton's degradation of 3-Aminopyridine	Karale et al..,	
2014	Study on the effect of addition of potassium, ammonia on the formation of type of ironhydroxy sulfates	Jones et al.,	
2016	Molecular studies on five different iron oxidizing bacterial strains	Latorre et al.,	Model on Metabolic Pathway
2017	Synthesized iron hydroxysulphates jarosite & schwertmannite and their catalytic role in Fenton's degradation	Yan et al.,	
2019	Bacteriological synthesis of iron hydroxysulfate using an isolated *Acidithiobacillus ferrooxidans* strain and its application in ametryn degradation by Fenton's oxidation process.	Bhaskar et al.,	➢ Isolation and Characterization of *A. ferrooxidans.* ➢ Biosynthesis of iron hydroxy sulphate. ➢ Application of jarosite in Fenton's oxidation

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CHAPTER 3

MATERIALS AND METHODS

The general steps followed in the current research are illustrated with a flow chart in the figure 3.01.

3.1 MATERIALS AND REAGENTS

3.1.1 Materials

Chemicals used for the experiments ammonium sulfate, magnesium sulfate heptahydrate, dipotassium hydrogen phosphate, potassium chloride, calcium nitrate, ferrous sulfate heptahydrate, sodium hydroxide, silver sulfate, potassium iodide, mercuric sulfate, sulfuric acid, silver nitrate, 1,10 phenanthroline, sodium thiosulfate, ferrous ammonium sulfate, Tris-HCl, EDTA, SDS, PVP, LiCl, Tween 20, Tris base, Glacial acetic acid, Xylene Cyanol, Bromophenol blue, Gylcerol, Ethidium bromide, Agarose, Titanium dioxide, Hydrogen peroxide (30 %) were purchased from Merck, India.

3.1.2 Reagents

Lysis buffer, Tris-saturated phenol, Chloroform, NaCl, EDTA, Tris-HCl, 10X PCR buffer, $MgCl_2$, dNTPs, (dATP, dCTP, dGTP, dTTP)*Taq* DNA polymerase, oligo-primers, milliQ water, agarose gel, 1X TAE buffer, ethidium bromide, DNA loading dye, standard DNA marker. All reagents were prepared according to the protocols of Sambrook and Russell, (2001).

The necessary glassware and other materials used in the present study were either wet sterilized or dry sterilized. Wet sterilization was achieved at 121 °C for 20 min in an autoclave. Dry sterilization was performed at 180 °C for 4 h in a hot air oven. Other requirements included sodium hypochlorite solution (1%), Stereobinocular Microscope, Compound/Bright field Microscope, polythene bags, Petri plates, microslides, microslips, stains, needles etc. Molecular studies required eppendorf tubes, Microcentrifuge (Eppendorf), Surecycler 8800 (Agilent Technologies, India), Submarine electrophoresis unite and Gel documentation system (Vilber Lourmat) etc.

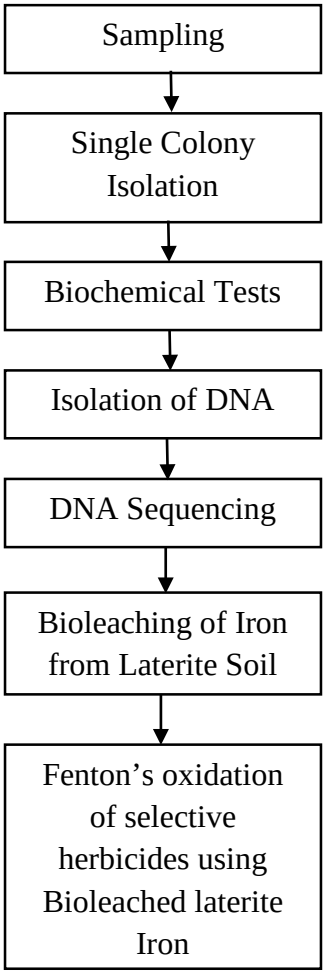

Figure 3. 01. Flow chart dissipating the methodology

3.2 SAMPLING

3.2.1 Acid Mine Drainage

AMD samples were collected from operating coal field at margarita, Assam, India as shown in the figure 3.02. Open cast mining was employed in the selected field. Mining waste water were collected, stored and treated with neutralization and other techniques prior to the discharge into nearby stream to meet pollution control board standards. Four points were selected for the sampling based on field observation. Sampling points are shown in the figure 3.03. The first sample was collected at site near to the generation of drainage water where pH was found to be more acidic. Second collection point was along the drainage flow whereas the third collection point was at the storage tank. Fourth sample was from the discharge pipe at the collection tank. Prior to sample

collection the site was inspected for onsite temperature and pH at each sampling points. 45 cc syringes were used to draw the sample from drain. Samples drawn were collected in the sterilized glass bottles and transported to the laboratory packed in ice.

Figure 3. 02. Location of AMD sampling site

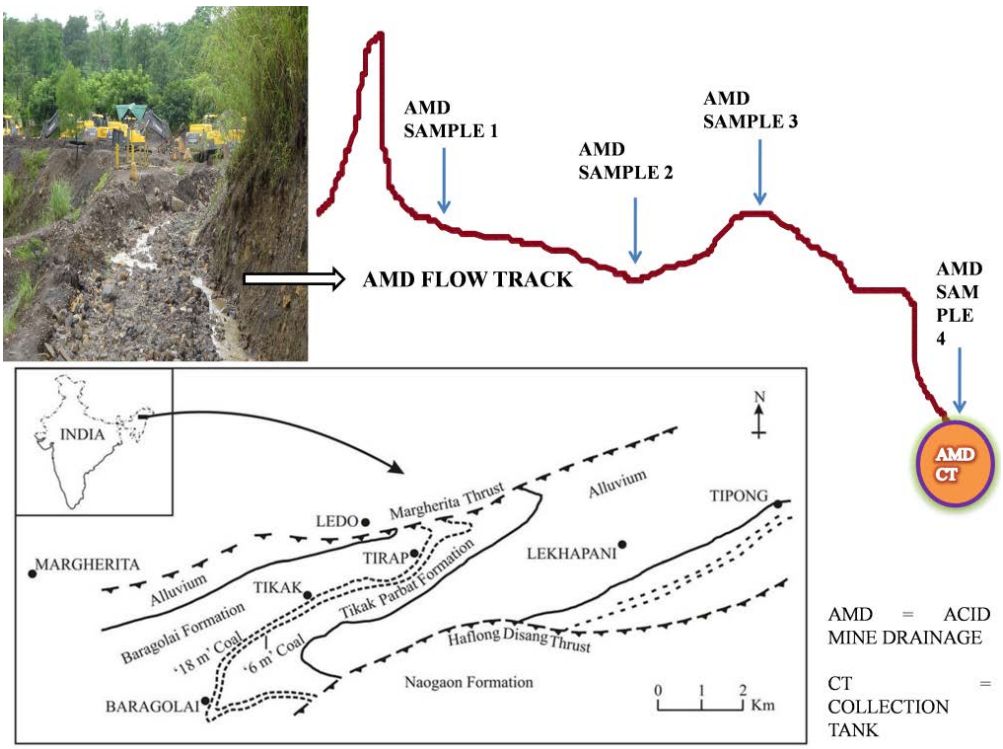

Figure 3. 03. Sampling points on AMD

3.2.2 Lateritic soil sample

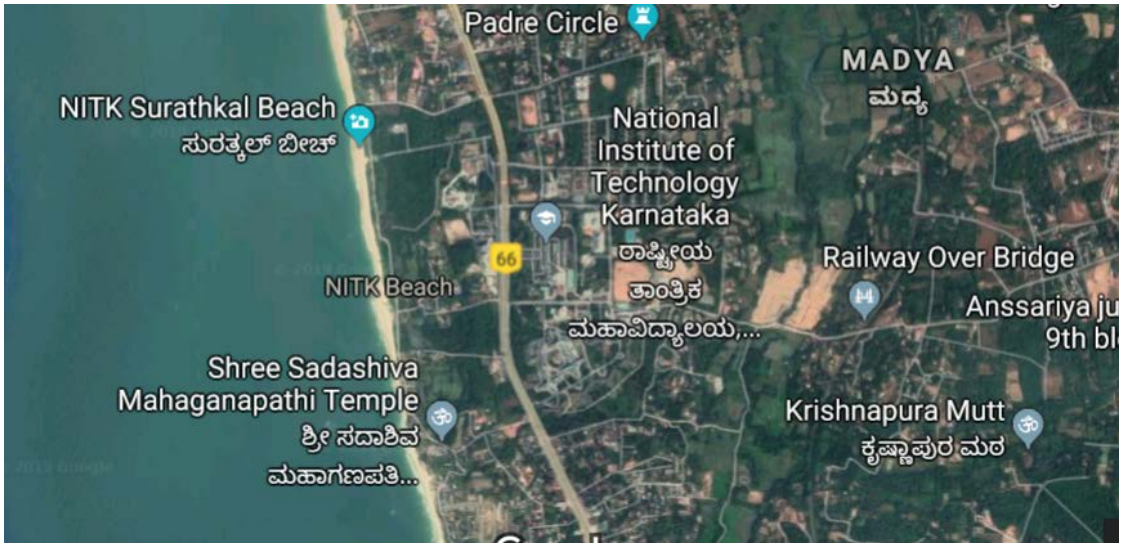

Figure 3. 04. Location of Lateritic soil sampling site

Study soil for the bioleaching was collected from the NITK campus located in Mangalore. Samples were collected at a depth of 1 meter to ensure erosion free soil samples. Samples collected were crushed into finer particles, sieved and analyzed for its morphological, mineralogical and chemical characteristics.

3.3 ISOLATION OF IRON OXIDIZING BACTERIA

3.3.1 Isolation and Screening of Iron oxidizing bacteria

AMD samples collected were inoculated in the enrichment media for isolation. Isolation media used is modified 9K media composition of which is shown in the table 3.01 (Wood and Don. 1993). Solution A of the medium was autoclaved except solution B which was filter sterilized before mixing to avoid precipitation on heating. The medium was adjusted to pH 3 prior to sterilization. Under sterilized condition the media was inoculated with 4% AMD samples, incubated for 15 days in incubator shaker at 30^{o}C and at 180 rpm.

Table 3. 01. Composition of Modified 9K medium

Medium Composition	Quantity in g/L
Solution A	
Ammonium sulfate	3
Di potassium hydrogen phosphate	0.5
Magnesium sulfate heptahydrate	0.5
Potassium chloride	0.1
Calcium nitrate	0.01
Solution B	
Ferrous sulfate heptahydrate	44.22

Enrichments that showed active growth on incubation were diluted by serial dilution (1 x 10^{-6}) and spread into modified 9K solid media prepared with the same media composition with 2% agarose additional supplement for solidification by spread plate method. Inoculated plates were examined for the active growth and selected and were streaked into fresh solid medium repeatedly until a pure isolates were obtained. Screening was done based on the growth potential of iron oxidizing bacteria.

3.3.2 Identification of Isolates by 16S rRNA sequencing

3.3.2.1 Isolation of genomic DNA

Isolated bacterial cells of 10 – 12 days old culture were harvested by centrifuging 50 ml broth at 8000 rpm for 10 min. Cell pellets were washed twice with PBS and incubated for 60 min at 37^{o}C. The solution was added with 400 µl of lysis buffer and 15 µl of proteinase K and incubated for 60 min at 55^{o}C. The solution was centrifuged for 15 min at 4^{o}C with the supplement of 400 µl phenol:chloroform (1:1). The upper aqueous layer was taken out and 400 µl of chloroform:isoamyl alcohol (24:1) was added following the centrifugation at 10000 rpm for 15 minutes and incubation at -20^{o}C for 60 min. After incubation 5 µl of 5M NaCl and twice the volume of absolute alcohol were added to the collected supernatant. The mixture was centrifuged at 14000 rpm after 3 h to precipitate DNA. Precipitated DNA was rinsed with 70% alcohol, air dried and pellets were suspended in nuclease free water and stored at -20^{o}C for further analysis (Chennappa et al. 2014).

3.3.2.2 16S rRNA gene amplification by PCR

PCR amplification was carried out with universal primers, and species specific primers details of which is given in table 3.02 (Chen et al. 2009; Escobar et al. 2008; Hall et al. 1996; Taylor et al. 2014). PCR amplification with the universal primers was carried out to 50 µL volume with the following composition: 4 µL of DNA extract, 5 µL of PCR buffer, 5 µL of $MgCl_2$, 4 µL of dNTP's, 2 µL of 24F primer and 2 µL of 1492R primer, 0.25 µL of Taq polymerase and 27.75 µL of nuclease free water. The gene amplification process was programmed for initial denaturation with 94^{o}C for 5 min each cycle followed by 30 cycles, each cycle was conditioned 94^{o}C for 30 sec, 55^{o}C for 45 sec, 72^{o}C for 90 sec and final extension with 72^{o}C for 7 min. While the PCR amplification with species specific primers was carried out with the mixture of 0.5 µL dNTP's, 2.5 µL of Taq buffer, 1.5 µL of $MgCl_2$, 0.5 µL of Taq polymerase, 2 µL of DNA extract, 0.25 µL of F1 thio (sense) primer and 0.25 µL of R1 thio (antisense) primer, rus_F and rus_R (in case of rusticynin specific) to the final volume of 25 µL adding 17.7 µL of nuclease free water. The process was programmed for initial denaturation step with 94^{o}C for 5 min followed by 30 cycles, each cycle conditioned at 94^{o}C for 30 sec, 57 ^{o}C

for 45 sec, and 72 ºC for 90 sec and 72ºC for last 7 min. The PCR product obtained was run on ethidium bromide stained agarose gel prepared with 1.5% agarose immersed in 1X TAE buffer. The band formation was confirmed with gel doc instrument. The primers used in the study and corresponding annealing temperature are shown in Table 3.02.

Table 3. 02. Primers used in study and corresponding annealing temperature

Genes	Primers	Nucleotide sequence	Annealing temperature ºC	Target site	Reference
16s rRNA	27F	5' AGAGTTTGATCMTGGCTCAG 3'	55	16s rRNA	Chen et al., 2009
	1492R	5' GGTTACCTTGTTACGACTT 3'		16s rRNA	Chen et al., 2009
Species specific	F1_Thio (Sense)	5' ATGCGTAGGAATCTGTCTT 3'	57	120 - 139	Escobar et al., 2008
	R1_Thio (Anti sense)	5' GGACTTAACCCAACATCTCA 3'		1078 - 1097	Escobar et al., 2008

The sequencing was done for the amplicon of 16s rDNA using F1_Thio (sense) and R1_Thio (antisense) primers approximately 900bp. Sequence identity was compared to those in the NCBI database (http://www.ncbi.nlm.nih.gov/) using the basic alignment local alignment search tool (BLAST) for nucleotide sequences. Phylogenetic analysis was carried out by constructing phylogenetic tree with MEGA 5.1 software by neighbor joining algorithm. The nucleotide sequence was submitted to NCBI Genebank.

3.4 BIOSYNTHESIS OF IRON HYDROXY SULFATES

Jarosite, an iron hydroxyl sulfate mineral was bacteriologically synthesized in modified 9K medium with 0.144M ferrous iron supplement to get total volume of 1000 ml. Culture medium was adjusted to pH 3.0 by adding 1N H_2SO_4 prior to incubation. *Acidithiobacillus ferrooxidans* cells of 100 ml volume with the cell density of 1.0 x 10^7 cells/ml were inoculated to the media and incubated in shaker at 180 rpm with 30^oC for 12 days. The precipitate formed on iron oxidation was filtered using Whatman's filter paper No.4, washed with acidified water of pH 2 and dried in oven to get jarosite powder which was stored for further use. The precipitate formed was characterized by X-ray diffraction and SEM.

3.5 BIOLEACHING OF IRON FROM LATERITE SOIL

Shake flask studies has been conducted to evaluate bioleaching of iron from laterite soil using an isolated bacterial strain. Laterite soil samples collected from the NITK campus, Surathkal, Karnataka, India were processed for required size and sterilized by steam autoclaving. Modified 9K media was used for bioleaching studies without ferrous iron supplement to which laterite soil was added to makeup 100 ml of mixture (Song et al. 2008). 10 % (v/v) of an isolated *Acidithiobacillus ferrooxidans* BMSNITK17 (Accession No. MG27180) inoculums of 1.0 x 10^7 cells/ml was added to this mixture to initiate the leaching studies (Bhaskar et al. 2019). Experiments were conducted varying operating conditions to study the effect of influencing parameters like Pulp density, Temperature, Shake flask speed, pH and Particle size. All the experiments were conducted in dual with sterile conical flasks on incubator shaker. Total iron leached was measured continuously.

3.6 FENTON'S OXIDATION OF SELECTIVE HERBICIDES

3.6.1 Biosynthetic jarosite catalyzed Fenton's oxidation

Catalytic role of biosynthetic jarosite was investigated for the Fenton's degradation of both Ametryn and Dicamba. 5 mg/L of initial ametryn concentration and 100 mg/L of dicamba concentration was used to mimic the field concentration (Sangami and Manu 2016, 2018). Ametryn and dicamba solution prepared in the laboratory were taken in a conical flask to which of jarosite powder was added in an incremental rate. The solution was adjusted to pH 3 using 1N H_2SO_4 and allowed 10 minutes for proper mixing to ensure uniform distribution of jarosite powder in the solution prior to H_2O_2 addition. Investigation was conducted with experimental conditions of jarosite (0.1 g/L – 1 g/L) and H_2O_2 dosage (100 mg/L – 1000 mg/L). Samples were drawn at regular intervals for analysis. During sampling, each time 1 ml of sodium thiosulphate was added to arrest the reaction (Khan et al. 2009). All the experimental analysis was conducted in triplicates.

3.6.2 Bioleached laterite iron catalyzed Fenton's oxidation

Iron catalyzed Fenton's oxidation of ametryn and dicamba were carried out with 5 mg/L and 100 mg/L of initial herbicide concentration to mimic the field concentration. Ametryn and dicamba solution prepared in the laboratory was taken in a conical flask to which of bioleached laterite iron solution was added in an incremental rate. The solution was adjusted to pH 3 using 1N H_2SO_4 and allowed 10 minutes prior to H_2O_2 addition to ensure that the addition of H_2O_2 triggers the initiation of reaction. Investigation was conducted with experimental conditions of bioleached laterite iron (10 mg/L – 40 mg/L for dicamba and 3 mg/L – 6 mg/L for ametryn) and H_2O_2 dosage (100 mg/L – 400 mg/L for dicamba and 30 mg/L – 60 mg/L for ametryn). Samples were drawn at regular intervals for analysis. During sampling, each time 1ml of sodium thiosulphate was added to arrest the reaction. All the experimental analysis was conducted in triplicates.

3.7 SCANNING ELECTRON MICROSCOPY (SEM)

Morphological feature of isolated *Acidithiobacillus ferrooxidans* strain was studied with scanning electron microscopy. Bacterial cells were fixed at 5^{o}C for 90 min with 5% gluteraldehyde in acidic buffer pH 2.5 stained with 0.25% uranil acetate at 4^{o}C for 1 h and air dried before examination. The morphology of biosynthesized jarosite was studied with the oven dried powdered precipitate. Also the morphological feature of oven dried laterite soil before and after bioleaching was studied with SEM. Change in structural and mineralogical composition during the leaching was studied. The sample were mounted on aluminum stub using double sided carbon tape, sputter-coated with gold and visualized using a S-3400N scanning electron microscope (Hitachi, Japan).

3.8 ANALYTICAL METHODS

Herbicide concentration were measured by a high-performance liquid chromatograph (HPLC) employing an Agilent 1200 with a C18 reverse phase column (pore size 3.5 μm ,100 × 0.46 cm), using water and methanol (in the ratio of 58:42) as mobile phase injected with a flow rate of 1.0 mL/min employing diode array detector (DAD). The volume of sample used for the measurement was 20 μm with retention time of about 10min (Sangami and Manu 2017). Chemical oxygen demand (COD) measurement was by colorimetric method as per 5220D of Standard Methods for Examination of Water and Wastewater (APHA Method 4500-F: 1992). The H_2O_2 consumption was measured using UV-Vis spectrophotometer (Eisenberg 1943). Total iron concentration was measured by 1,10 phenonthroline method and concentration of ferric iron was measured by KCN method using UV-Spectophotometer (Woods J., T and Mellon, M. 1941). Chloride concentration was measured by argonometric method of measurement (APHA Method 4500-F: 1992). The pH was monitored by digital pH meter (HANNA make). Redox potential was measured with ORP meter (Thermo Fisher make). For intermediate identification, samples after submitting to the degradation processes were analyzed by electrospray ionization/mass spectrometry using a positive electrospray ionization mode (ESI–MS), and a LC/MSDTOF (Agilent Technologies) mass spectrometer operated with the following parameters: capillary 4500 V; nebulizer 3.0

bar; drying gas 12.0 L/min; gas temperature 180 °C. Spectra were acquired over m/z 20–1000 range.

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CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF IRON OXIDIZING BACTERIA

4.1.1 Isolation of Iron oxidizing bacteria:

Onsite pH and temperature measured at the site during sampling were 3.0 and 30ºC respectively. Bacterial colonies showing active growth with high bacterial density in modified 9K media were selected for further characterization. Isolates were obtained from plated sample after 3 to 4 days of incubation. Dark reddish colonies obtained on plate morphologically confirmed the iron oxidation by the bacteria. About ten isolates were obtained from plated samples. Out of which three isolates exhibited growth on re-streaking and single pure isolate was finalized based on the growth. Isolates obtained were gram stained and confirmed the Gram -ve reaction. The cell morphology revealed that the strain is short and rod-shaped organism. The isolate showed efficient growth at pH 3 and at 30ºC temperature on modified 9K medium with ferrous being the energy source (Chen et al. 2009; Qin et al. 2013).

Table 4. 01. Morphological, Physiological, Biochemical Characterization of *A. ferrooxidans* BMSNITK17

Tests	***A. ferrooxidans* BMSNITK17**
Morphology	Rod
Gram Staining	-Ve
pH	3.0
Catalase	+ve
Oxidase	+ve
Oxidation of Iron	+ve
Growth Temperature	30 ºC
Energy Source	Ferrous

Among biochemical tests both oxidase and catalase tests showed positive results. Tests were evaluated in accordance with Bergy's Manual of Determinative bacteriology and are tabulated in Table 4.01. The isolate is chemolithiotrophic Gram -ve bacteria belonging to gamma proteobacteria (Ann, P., Wood, and Don, P. 1993).

4.1.2 Amplification, Sequencing and Identification:

Amplification using 27F and 1492R universal primer pair resulted in an amplicon size of 900bp. Also amplification using F1_Thio (Sense) and R1_Thio (Anti sense) a pair of species specific primer resulted in an amplicon size of 900bp. 16s rDNA sequence analysis of the isolate was identified as *Acidithiobacillus ferrooxidans* based on BLAST analysis. Dendogram representation of 16S rDNA based on BLAST algorithm was prepared and compared with previously published data. Phylogenetic analysis revealed that the isolated colonies are the members of acidophilic bacteria which are having autotrophic mode of metabolism. Isolates matched up to 99 % to the identified members. The optimal tree with the sum of branch length = 0.07797533 is shown Figure 4.01. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in units of number of base substitutions per site. The analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Wu et al. 2014). The characteristic features and nearest phylogenetic match is shown in Table 4.02.

Table 4. 02. Colony characteristics and nearest phylogenetic match

Isolate	Colony Morphology	*Nearest Match	Identity (%)	Isolation medium	Inferred Metabolism	Phylogenetic affiliation	Gen bank accession number
Acidithiobacillus ferrooxidans BMSNITK17	Rusty orange brownish appearance	*Acidithiobacillus ferrooxidans* ATCC23270	94	Ferrous sulphate	Iron oxidation/ chemolithotrophic	Gama proteobacteria	MG271840
		Acidithiobacillus ferrooxidans ATCC33020	93				

* Nearest relative to the isolate obtained was determined by BLAST method.

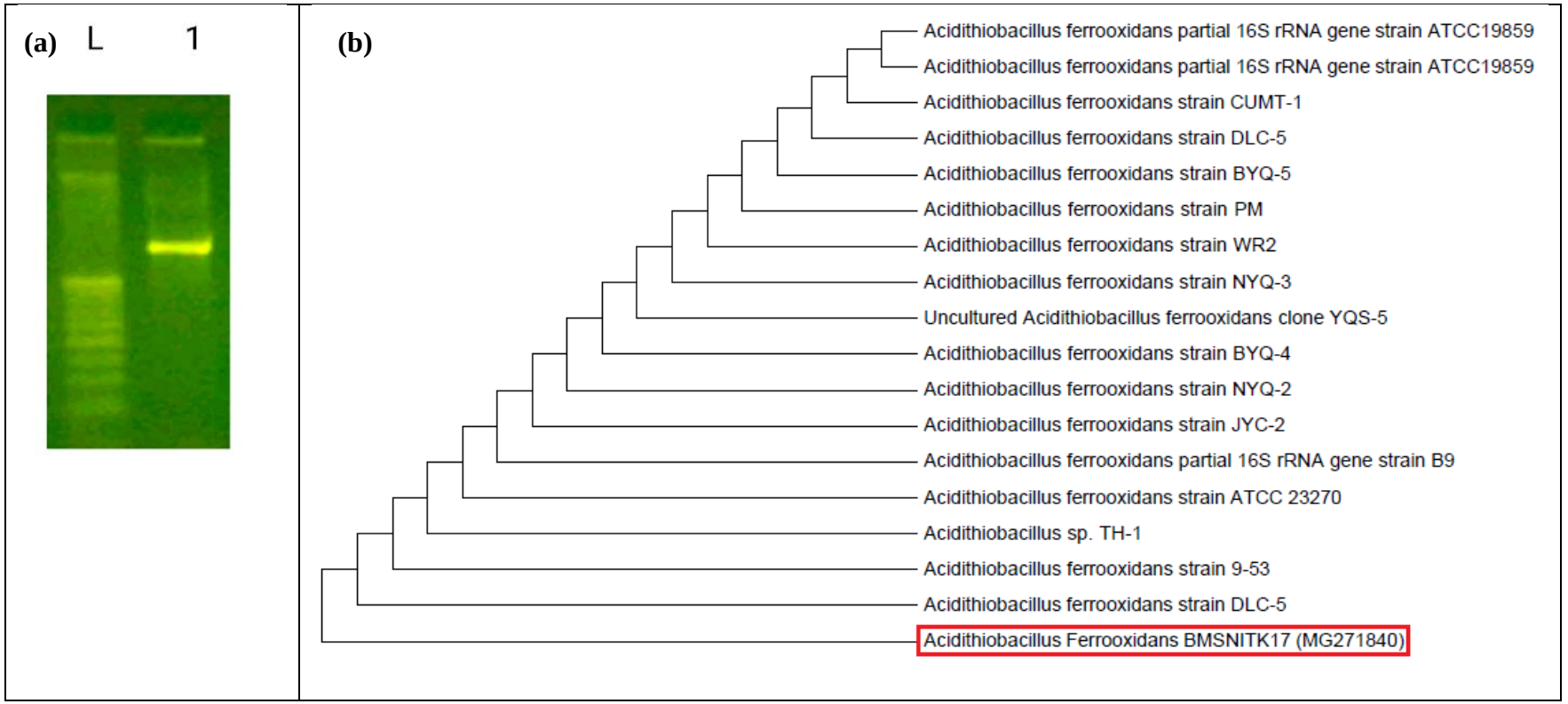


Figure 4. 01. a) Agarose gel showing an amplicon of 16s rRNA of the isolate, L – 100bp ladder, 1 – *Acidithiobacillus ferrooxidans* BMSNITK17 b) Dendogram representation of phylogeney of *Acidithiobacillus ferrooxidans* obtained. Strain belong to this study is shown highlighted in the box. Parenthesis number is obtained Genebank number.

The isolated bacterial strain is closely related to *Acidothiobacillus ferrooxidans*, a chemolithotrophic gamma proteobacteria and the sequence of the isolate was deposited in GenBank and the isolate has been assigned the accession number MG271840 by GenBank.

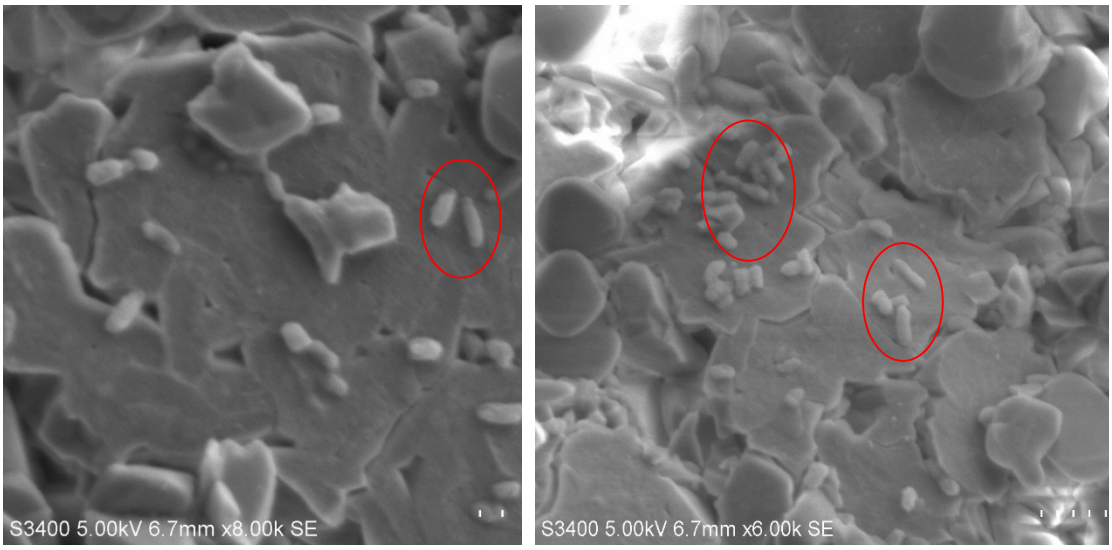


Figure 4. 02. SEM images showing *Acidithiobacillus ferrooxidans* BMSNITK17

4.1.3 Sequence Result of Universal Primers:

1B_27F-
E02.ab1GGGAGGGGGCTACATACCATGCAGTCGTACGGGGCAGGCACTTCGGATGCTGACGAGTGGCG
AACGGGTGAGTAATGCGTAGGAATCTGTCTTTTACTGGGGGACAACCCAGGGAAACTTGGGCTAATACC
GCAGGAGCCCTGAGGGGGAAAGCGGGGGATCTTCGGACCTCGCGCTAAGAGAGGAGCCTACGTCCGATT
AGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCAACGATCGGTAGCTGGTCTGAGAGGACGACCAGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCGGTGGGGAATTTTTCCCAATGGGGGCAA
CCCTGACAAAACAGTGCCCCGTGGATGAAGAAGGCCTTCGGGTTGTAAAGTCCTTTCGTGGAGGACAAA
AAGGTGGGTTCTAATACAATCTGCTATTGACGTGAATCCAAGAAGAAGCACCGGCTAACTCCGTGCCAG
CAGCCGCGGTAATACGGGGGGTGCAAGCGTTAATCGGAATCACTGGGCGTAAAGGGTGCGTAGGCGGTA
CGTTAGGTCTGTCGTGAAATCCCCGGGCTCAACCTGGGAATGGCGGTGGAAACCGGTGTACTACAGTAT
GGGAGAGGGTGGTGGAATTCCAGGTGTAGCGGTGAAATGCGTATAGATCTGCAGGAACATCAGTGGCGA
AGGCGGCCACCTGGCCCAATACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCC
TGGTAGTCCACGCCCTAAACGATGAATACTAGATGTTTGGTGCCTAGCGTACTGAGTGTCGTAGCTAAC
GCGATAAGTATTCCGCCTGGGAAGTACGGCCGCAGGTTAATACTCAAAGGAATTGACGGGCGCCCGCAC
AAGCGGTGGAGCATGTGGTTTATTTCGATGCAACGCGAAGACCTTACCTGGGCTTGACATGTCCGGAAT
TCTGCAGAGATGCGAAGTGCCCTTCGGGGAATCGGAGCACAGGTGCTGCATGGCTGTCGTCAGCTCGCG

TCTGTAATGTTGGGTTAAGTCCGCAACCAGCACAACCCTTGTTCTTACTGCCACGGATTCGGCCGACAC
TCTCAGGCAACTGCGCGACACCCGAGGAACGCGGAATAGATGAGTGCTCATGCCTTACTCCGGGGCTCT

1B_1492R-
F02.ab1GCGGGCGGTCACTCTCTAGGTACCGCCCTCCCGATGGTTACTCTGCTGCTTCTGGTGAATCC
ACTCCCATGGGGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCG
ATTACTAGCGATTCCCACTTCATGCAGTCGAGTTGCAGACAGCGATCCGAACTACGACACGCTTTCTGG
GGTCTGCTCCACCTCGCGGCTTGGCTTCCCTCTGTACGCGCCATTGTAGCACGTGTGTAGCCCTGGACA
TAAAGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCCTAGAGTG
CCCGGCCGAACCGCTGGCAACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACG
ACACGAGCTGACGACAGCCATGCAGCACCTGTGTTCCGATTCCCCGAAGGGCACTTCCGCATCTCTGCA
GAATTCCGGACATGTCAAGCCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCG
CTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAACCTTGCGGCCGTACTTCCCAGGCGGAATACTTA
TCGCGTTAGCTACGACACTCAGTACGCTAGGCACCAAACATCTAGTATTCATCGTTTAGGGCGTGGACT
ACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCAGCGTCAGTATTGGGCCAGGTGGCC
GCCTTCGCCACTGATGTTCTCTCCAGATCTCTACGCATTTCACCTGCTACACGTGAAATTCCACCACCC
TCTCCCATACTCTAGTACACCGGTCTTCCACCGCCATCTCCAAGGTTGAGCCCGGAGATTTCACGACAG
ACGTACGTACCGCCTACGCACCCTTTACGCCCAGTGATTCCGATAACGCTTGCACCTCGTATTACCGCG
GCTGCTGCACGAGAGTTAGCCGATGCTTCTTCGTGGATCACGTCAATACAGACTCGTATAGACATACAT
ATCGTACTACATGAAGACTTACAACCTGAGCATCGTCATCACCGTCATGCATCTCAGGTGCCCCATGGA
AGATCACACTGCTGCTCCCGAGGAGCTGGACTGTTCTATCAGTGGAGTGGCGGCTCTTCGAGACATACA
TCCACTGTGGAGCTGAGCCGCACTAGCTATCGGAATGCCCTCTGTACCGGGATGCAG

4.1.4 Sequence Result of Universal Primers:

B1_F1-
A06.ab1AGATTGGTAATTTTAGAATAGTCTGTGTACTTTTGCCAAAGGGTCTCAAAAAAATTTAAGGC
ACCTGCATAAAAAGAGGCTCCCGTTACCGCCTCGCTTTGTACTTTTTCTTGTTACCTGCCGTTATTGGA
GAGAGGAGCACTTTTTTTCTTTCGTTACCCTTCCCTCCTTTGTGCCTTGAAATCCCTCTCGACTACCCC
TCGATTTTCTCTTTCTACCTCGGGTAAGGCCGGCCTGCACCCCCCCCGTGAACCTTACCTGGGAGGTCC
CTACAAGGAAAACTACCCGTGCGCCCAACCCGCCAAAGACGACATCATCATGCCCAATGAAATT

4.2 BIOSYNTHESIS OF IRON HYDROXY SULPHATE:

On biooxidation of ferrous sulphate by *Acidithiobacillus ferrooxidans* BMSNITK17 cell suspensions at pH 3, yellow iron precipitates were formed with the solution containing 0.144M ferrous sulphate and 0.06M potassium. Meraune and Vargas. (2003) observed that below pH 5 bacterial oxidation of ferrous is predominant over chemical oxidation (Meruane 2003). The rate of proton diffusion hinders below pH 2.5 as the

ferric oxide layer formed allows the accumulation of precipitates on the bacteria thus reducing the rate of oxidation. Huang et al., (1993) observed that the rate of oxidation of ferrous iron on mineral phase of jarosite precipitate depends on local pH changes (Huang and Zhou 2012). In the present study jarosite precipitation occurs within the initial 10 days of inoculation of bacteria into ferrous sulfate medium under low pH condition. No much variation in the pH was observed during biooxidation and the reason for this is attributed to changes in pH during the biooxidation of ferrous iron was hindered by the hydrolysis of ferric ions. At 0.144M iron concentration and 0.06M potassium concentration in the medium yellow precipitate is formed. The formed jarosite precipitates are ternary solids with monovalent cation K^+ (Wang et al. 2006).

$$3Fe^{3+} + K^+ + 2SO_4^{2-} + 6H_2O \longrightarrow KFe_3(SO_4)_2(OH)_6 + 6H^+ \quad \text{(Eqn xiv)}$$

During the initial four days, the biooxidation rate was slightly high and this may be due to the formation of ferric iron prior to jarosite precipitate under low pH condition. On biooxidation of ferrous sulphate by the *Acidithiobacillus ferrooxidans*, the formation of jarosite precipitation was less in quantity. Iron precipitate attaches to the extracellular polymeric substances (EPS) during the biooxidation and also formation of EPS-Fe^{3+} complex lowers the precipitate formation quantitatively (Huang and Zhou 2012). Less jarosite precipitate formation can be attributed to the drop in pH to less than 2 in seven days (Daoud and Karamanev 2006). Iron precipitate appears to be agglomerated particles resembling rose petals with smooth interface through SEM images observed (Figure 4.03). The XRD analysis of particles exhibits six characteristic broad peaks 24.44, 29.14, 35.15, 39.47, 47.74 and 50.04 at an angle 2Ɵ with unit cell parameter *c* being 17.034 A^o. Signal to background ratio of XRD patterns in figure 4.04 showing lowest ratio indicates its poor crystallinity (Liu et al. 2003; Wu et al. 2016). The formed precipitates are said to be jarosite (PDF No 01-071-1777). The formation of jarosite with ferrous iron supplement in presence of potassium is inconsistent with the previous studies with broad peaks observed at 2Ɵ matching to yan et al., (2017). Specific surface area of the synthesized biogenic jarosite is 1.62 m^2/g as per BET analysis. Elemental composition and specific surface area reveals that the jarosite is formed directly on biooxidation of ferrous sulphate medium provided (Qiu et al. 2006).

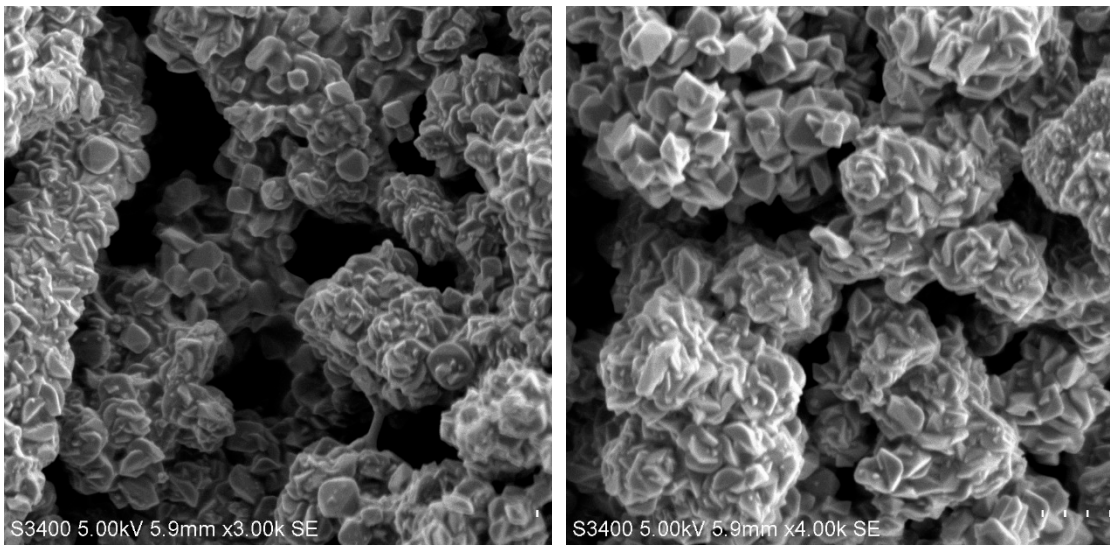


Figure 4. 03. SEM images showing Biosynthetic Jarosite

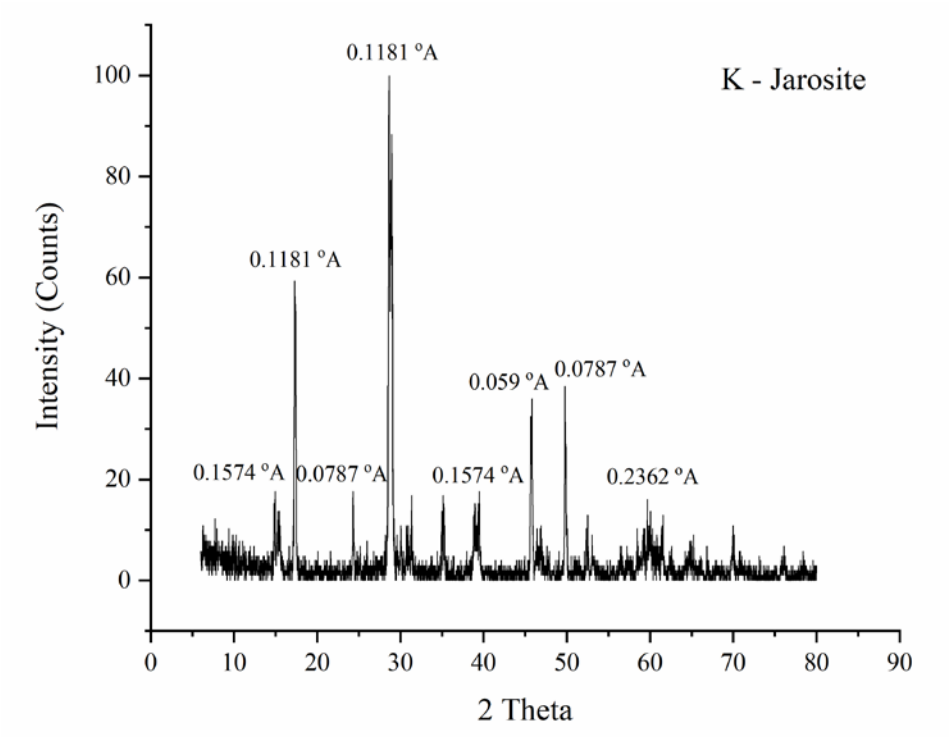


Figure 4. 04. XRD Plot of Biosynthetic Jarosite

4.3 BIOLEACHING OF IRON FROM LATERITIC SOIL

4.3.1 Characterization of Fresh Lateritic

Fresh laterite soil was examined for its chemical composition and also for morphological appearance. Table 4.03 gives the chemical composition of fresh laterite soil. Figure 4.05 represents SEM images of fresh laterite soil and figure 4.06 gives the XRD representation of fresh and bioleached laterite soil. Fresh lateritic soil contains 40.18 % of iron oxide indicating the richness of study soil with iron. XRD plot indicates the presence of silica as quartz, and iron oxide. The fresh lateritic soil particles on SEM appear to be amorphous, fibrous like structure. The study soil exhibits the presence of quartz (PDF No. 01-078-1254), and Iron oxide (PDF No. 00-001-0662) with broad peaks at 2Ɵ 26.81, 50.27, 55.09, 57.93, 24.32, 57.93, 64.52. EDS plot shown in figure 4.07 indicates the representative peaks for the presence of oxygen, iron, aluminum, nickel, cobalt and magnesium.

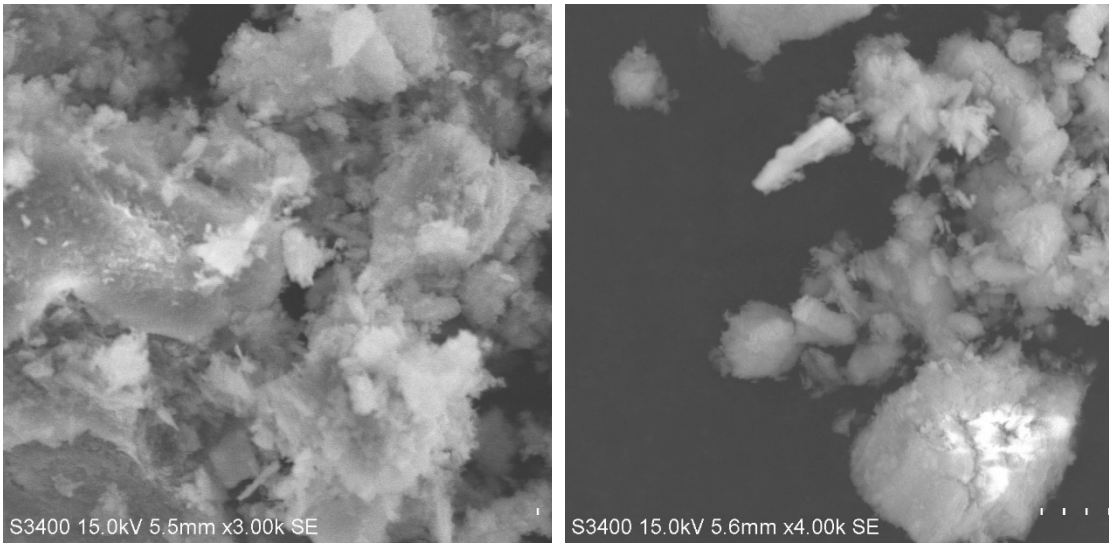


Figure 4. 05. SEM Images showing Fresh Lateritic Soil

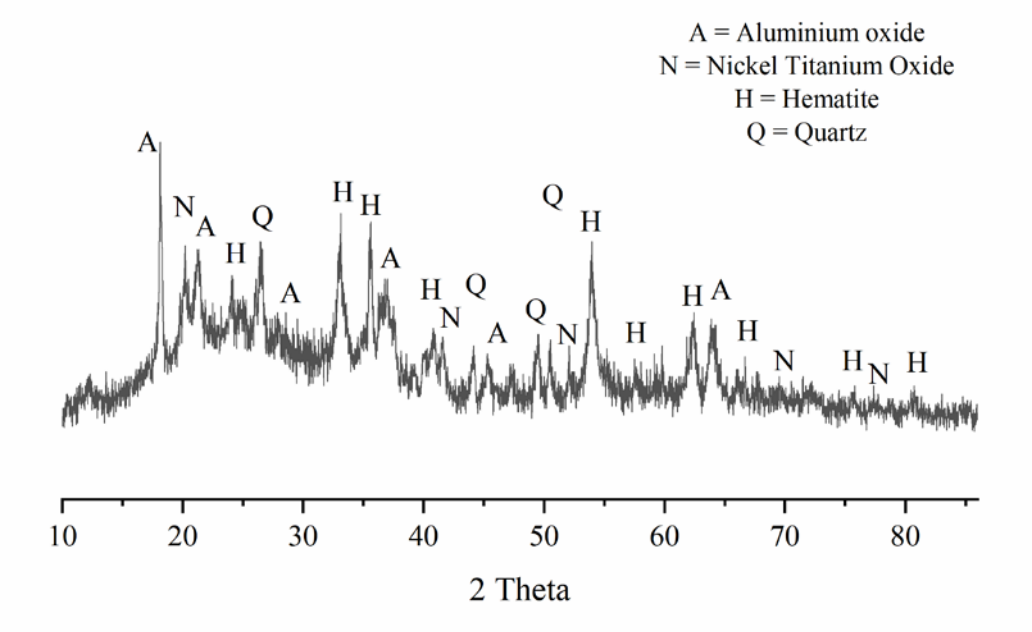


Figure 4. 06. XRD Plot of Fresh Lateritic Soil

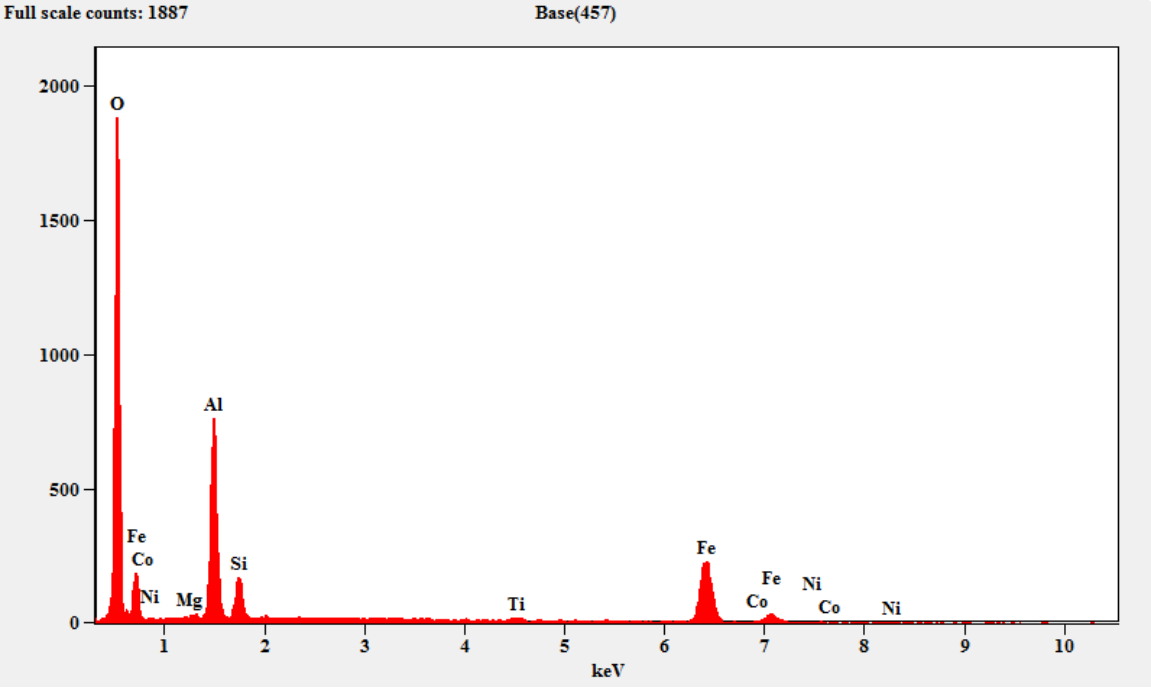


Figure 4. 07. EDS Spectrum of Fresh Lateritic Soil

Table 4. 03. Chemical composition of Fresh Lateritic soil

Components	Percentage (%)
Aluminium Oxide (Al_2O_3)	4.98
Iron Oxide (Fe_2O_3)	40.18
Silica (Si_2O_3)	36.07
Others	18.77

4.3.2 Effect of shake flask speed:

Agitation speed in the bioreactor is of much concern of process engineering which corresponds to shake flask speed at lab scale shake flask bioleaching studies. In the present study the effect of the shake flask speed at different variants like 100 rpm, 180 rpm and 250 rpm was studied while the other parameters kept constant i.e pH 3, Pulp density 5%, temperature 30 ^oC, 150µm. At 180 rpm the rate of dissolution was comparatively high since the agitation at this speed holds the bacterial suspension in contact with ore allowing ore particles not to settle down at the bottom. In unbaffled shake flask it is the flow pattern which affects the rate of leaching (Büchs 2001). Buchs's (2001) study confirms that the laminar flow at 100 rpm and 180 rpm keeps the suspension in contact with bacteria while at 300 rpm the rate of dissolution is low because of turbulence occurs at this speed. Higher shear force at high speed of shake flask might be one of the causes for cell detachment to the ore surface and cell rupturing (Brock 1978).

Maximum iron dissolution of 258.3 mg/L was observed at 180 rpm on fourth day of leaching studies. Figure 4.08 presents the rate of iron dissolution at different shake flask speed. Iron dissolution at 180 rpm reaches maximum whereas the leaching experiments with 100 rpm and 250 rpm shows resistance to iron dissolution at a considerable rate. Vegilo and team (1997) proposed a model for oxygen mass transfer rate in shake flask and claims that stirring had a positive effect on the oxygen transfer rate (Veglio et al. 1998). The graph shows there was a significant change in iron dissolution at 180 rpm compared to 100 rpm and 250 rpm. The rate of iron dissolution was increased by 37.1 mg/L on second day, 27.9 mg/L on third day and 41.2 mg/L on the fourth day. There was a decrease in iron concentration in the solution on later day. The reason attributed

for this is the precipitation of ferric iron due to hydrolysis and the maximum utilization of ferrous iron leached out from the laterite by the bacteria.

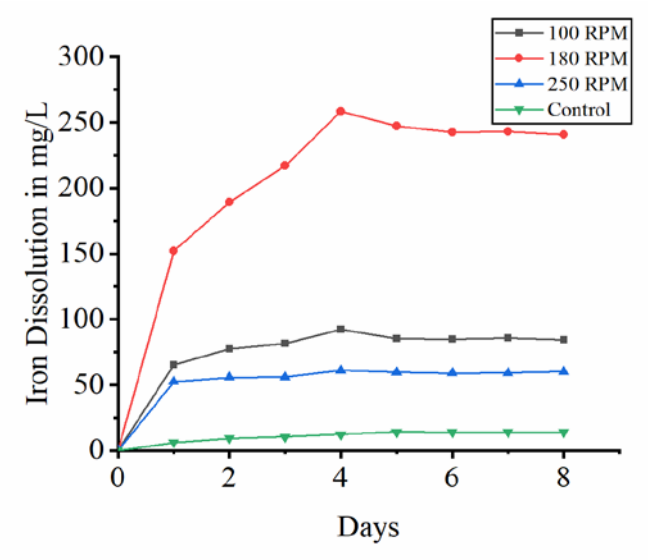


Figure 4. 08. Iron Dissolution on Bioleaching under different shake flask speed.

Figure 4.10 gives the redox potential and pH variation during the experimental investigation. On observing the graphical representation the redox potential varies in between 550 to 600 mV. There was a sudden rise in the redox potential from the first day to second day of inoculation representing the bacterial oxidation activity contributing to high ferrous to ferric ratio. After fourth day there was a slight drop in redox potential which remains almost constant till the eight day. Redox potential at 180 rpm reaches to its maximum of 620 mV by the fourth day. After the fourth day there was a sudden decrease in the redox potential indicating the bacteria has reached its stationary phase. Redox potential of other flaks lies between 500 mV to 550 mV throughout the studies. It is because of this oxidizing environment redox potential is positive throughout the studies.

There was a constant drop in the pH value in all flasks dropping to below 2.5 by fourth day and there was a slight increase in the pH indicating the lower bacterial activity. This similar pattern of pH was observed both in shake flasks speed of 100 rpm and 180 rpm. With the shake flask speed of 250 rpm the pH value decreases to 2.5 till the fourth day and thereafter there was a rise in pH to 2.9. This confirms that the bacterial activity existed till fourth day was hindered after due to the death of bacteria.

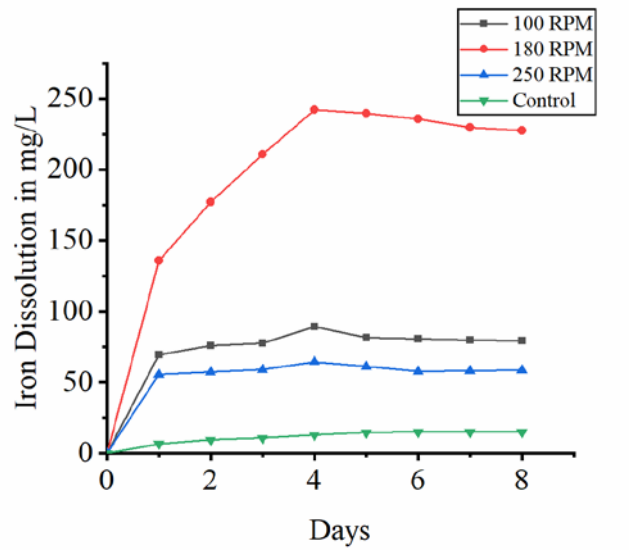


Figure 4. 09. Repeated Bioleaching experiments at different shake flask speed.

Control experiment without inoculation shows there was no significant leaching in the absence of the bacteria and the pH value maintained remains almost constant throughout the study. Iron dissolution in control set was less than 2 % compared to the bioleaching experiments. This confirms the role of bacteria in leaching out iron from lateritic soil.

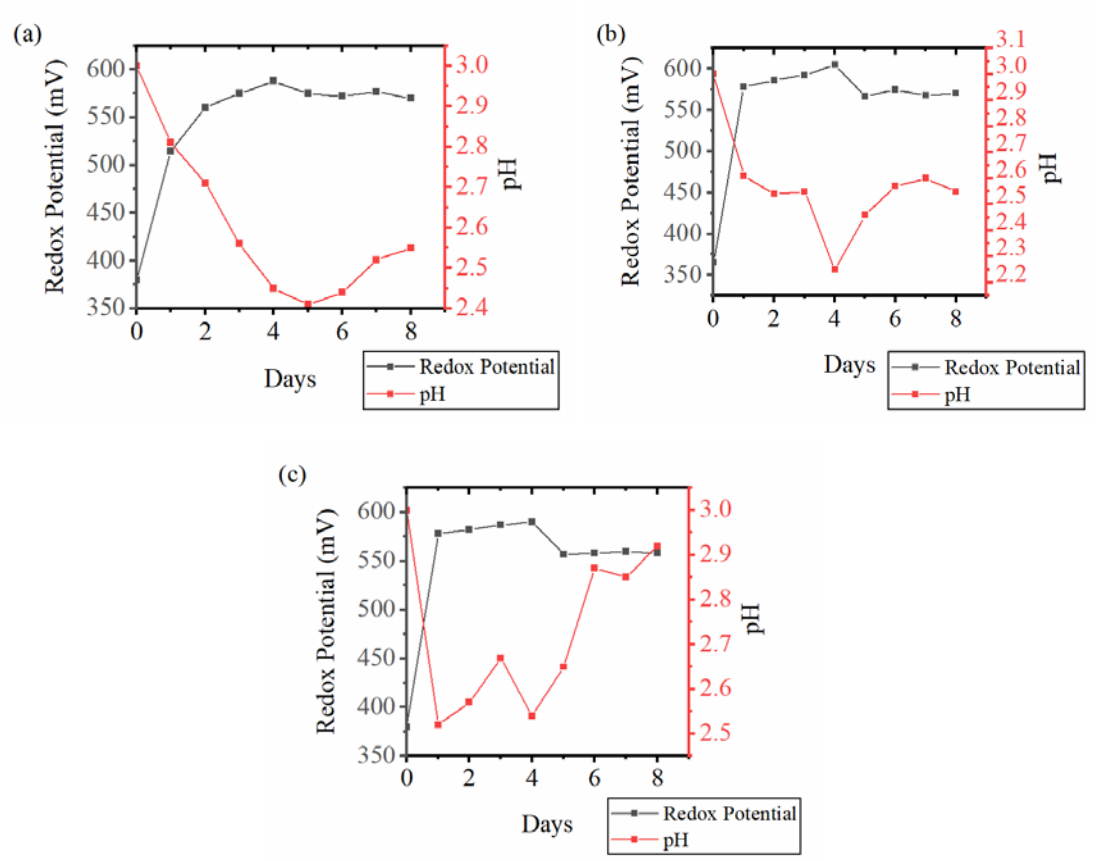


Figure 4. 10. Variation of Redox potential and pH at different shake flask speed on first experimental investigation (a) 100 rpm (b) 180 rpm (c) 250 rpm.

Bioleaching of iron from lateritic soil under different shake flask speed was investigated on repetition for the confirmation. Figure 4.09 represents the iron dissolution at different shake flask speed on first repetition. The results follow the same pattern as in case of first investigation showing its maximum leaching at 180 rpm. Figure 4.11 represents the redox potential and pH variation on repetition. The results obtained from the repeated experiments are inconsistent with first investigation.

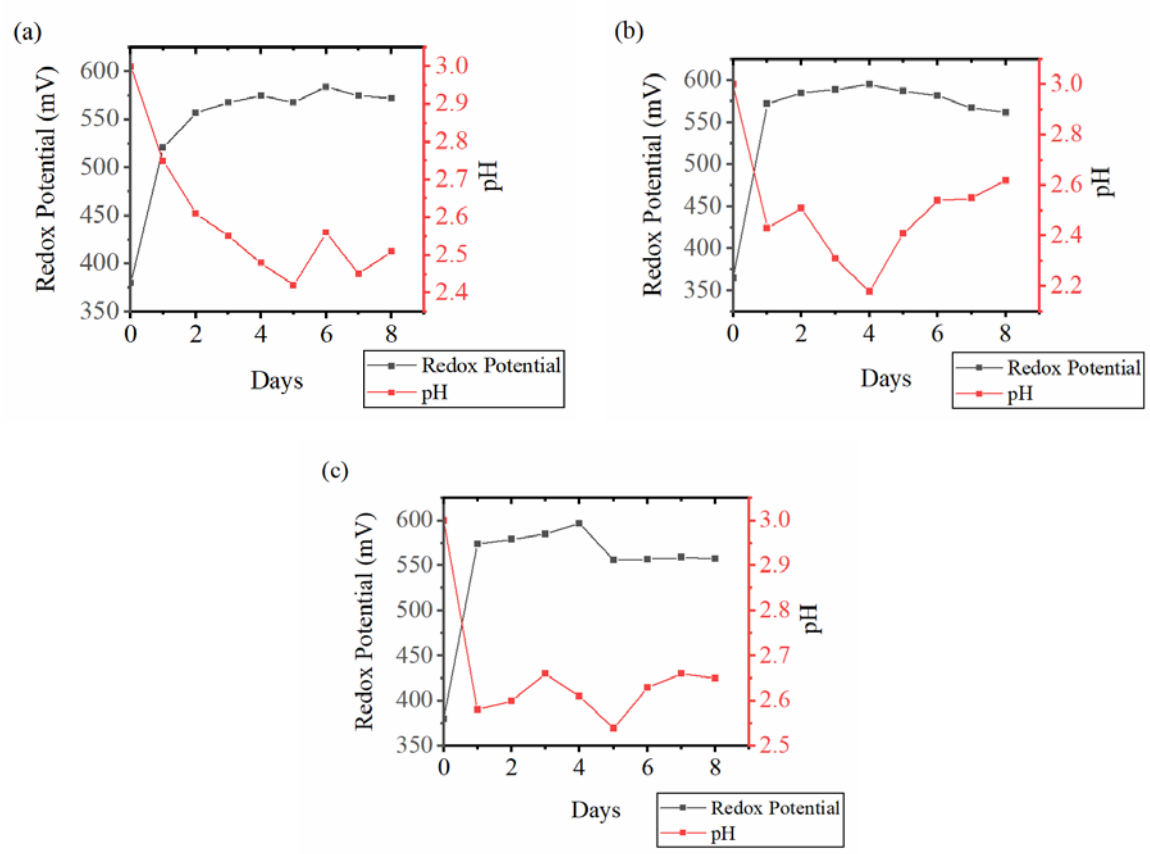


Figure 4. 11. Variation of Redox potential and pH at different shake flask speed on repeated experimental investigation (a) 100 rpm (b) 180 rpm (c) 250 rpm.

4.3.3 Effect of pH:

pH is the prime factor for both microbial growth and biooxidation. Bioleaching of iron from laterite soil under different pH was evaluated while the other parameters kept constant i.e shake flask speed 180 RPM, Pulp density 5%, temperature 30 ^{o}C, 150µm. pH value in the range of 1.5 to 3.0 gives highest biooxidation rate (Deng et al. 2000). In the present study higher redox potential in the range of 550 – 600 mV supports this statement. Fowler and coworkers (1999) claim that even on reduction of pH from 1.7 to 1.3 the rate of iron dissolution increases. This might be due to chemical dissolution of iron into solution at lower pH (Fowler et al. 1999). Figure 4.12 demonstrates the iron dissolution from lateritic soil at different pH. In the present study maximum iron dissolution of 281.0 mg/L was observed at pH 2.5. Thus the result obtained is within the optimum range of pH for bacterial metal dissolution process. In the first four days of leaching initial drop in pH was observed. This drop in pH is due to ferrous oxidation

resulting in more proton consumption (Acevedo and Gentina 1989; Blázquez et al. 2003; Klaus 1997). Caranza and Paleneia (1996) claim that pH lower than 0.8 inhibits bacterial growth in spite of acidophilic mode of habitation in leaching studies (Palencia et al. 1998). Battagalia and his coworkers (1994) stated that bacterial growth is usually inhibited at pH 1.5 (Battaglia et al. 1994). In contrast to this, in the present study we observed a significant iron dissolution rate at pH 1.5. The reason for this is attributed to the predominant chemical oxidation at lower pH in favor of oxidizing environment which accounts for iron leaching rather than bioleaching. To check this uninoculated control was kept at pH 1.5 in which dissolution of iron was observed confirming the leaching of iron in small quantity. At pH 3.0 the rate of leaching hinders after first two days. The reason attributed to this is ferric precipitation at higher pH due to hydrolysis prevents the bacterial cell attachment to the soil particle surface. Initial decrease in pH during the study indicates the biooxidation of iron while the later increase indicates ferric iron precipitates by hydrolysis and also shift in iron oxidation to sulfur oxidation (Stott M. B and Watling 2000). Drop in pH may also be due to bacterial growth whereas increase in pH indicates no further cell growth.

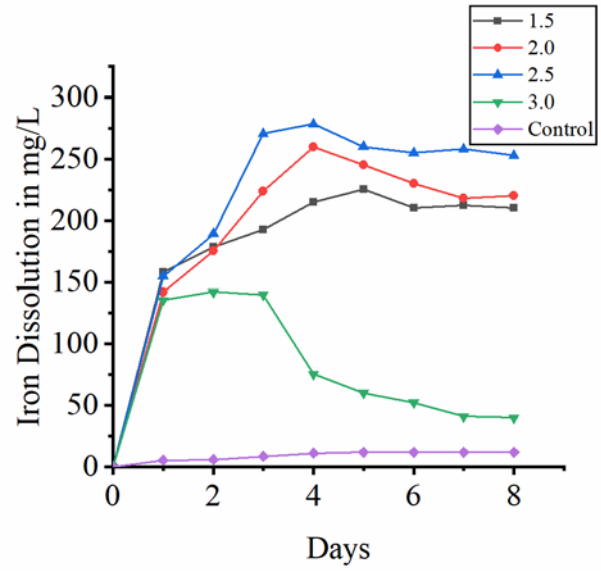


Figure 4. 12. Iron Dissolution on Bioleaching under different pH.

Figure 4.12 demonstrates the rate of iron dissolution varies at different initial pH maintained. This pH serves as optimum for bacterial action. Experimentation with pH 3 reveals that the bioleaching activity is not much favorable for bacterial leaching.

There might be formation of ferric hydroxide precipitates that hinders the bacterial accessibility to mineral for leaching at higher pH. On observation pH 2 - 2.5 is suitable range for bacterial iron leaching whereas with pH 3 iron dissolution falls after third day with drop in rate of iron leaching 11.7 mg/L per day at pH 2 and 13.075 mg/L per day at pH 2.5. Control set run without inoculation of bacterial strain shows insignificant leaching confirming the role of bacteria in iron leaching.

Figure 4.13 shows both redox and pH variation during the study. With all pH maintained there was a sudden increase in the redox potential which with slight variation reaches to maximum by third and fourth day indicating maximum leaching. In the present study the pH decreases in the first two days of leaching due to acid production on bacterial activity and increases later on indicating the ferric precipitation due to hydrolysis. The study soil laterite contains much ferric form of iron than ferrous which again contributes to jarosite precipitation thereby hindering the mass transfer of ions into the solution (Stott M. B and Watling 2000). Initial pH 1.5 maintained drops to 0.5 during the studies whereas with initial pH 2 and with pH 2.5 the pH drops to almost to 1.4. This drop in pH indicates the production of sulfuric acid due to bacterial activity. The rate of iron oxidation in the study is narrow within the narrow range of pH 2 to 2.5.

Redox potential reaches its maximum above 600 mV with pH 1.5 and pH 2.5. Redox potential shows increase in relation to decrease in pH from the first day to third day of study and there by remains constant. High iron content of laterite leads to high value of redox potential providing oxidizing environment in favor of bacteria. Since the rate of total iron dissolution does not alter significantly the redox value remains constant till the end of the study.

Repeated experiments conducted to investigate the effect of initial pH on bioleaching of iron confirm the effective range of pH for bioleaching operation as 2 and 2.5 (Figure 4.14). On repeat of experiments the drop in iron dissolution was observed with initial pH of 1.5 after fourth day. In consistent with this there was a drop in redox potential (Figure 4.15). The result obtained with initial pH 1.5 on repeat is in contrast with the

results obtained on first experimental investigation. The reason attributed to this was the halt in the bacterial activity. The pH increases after fourth day.

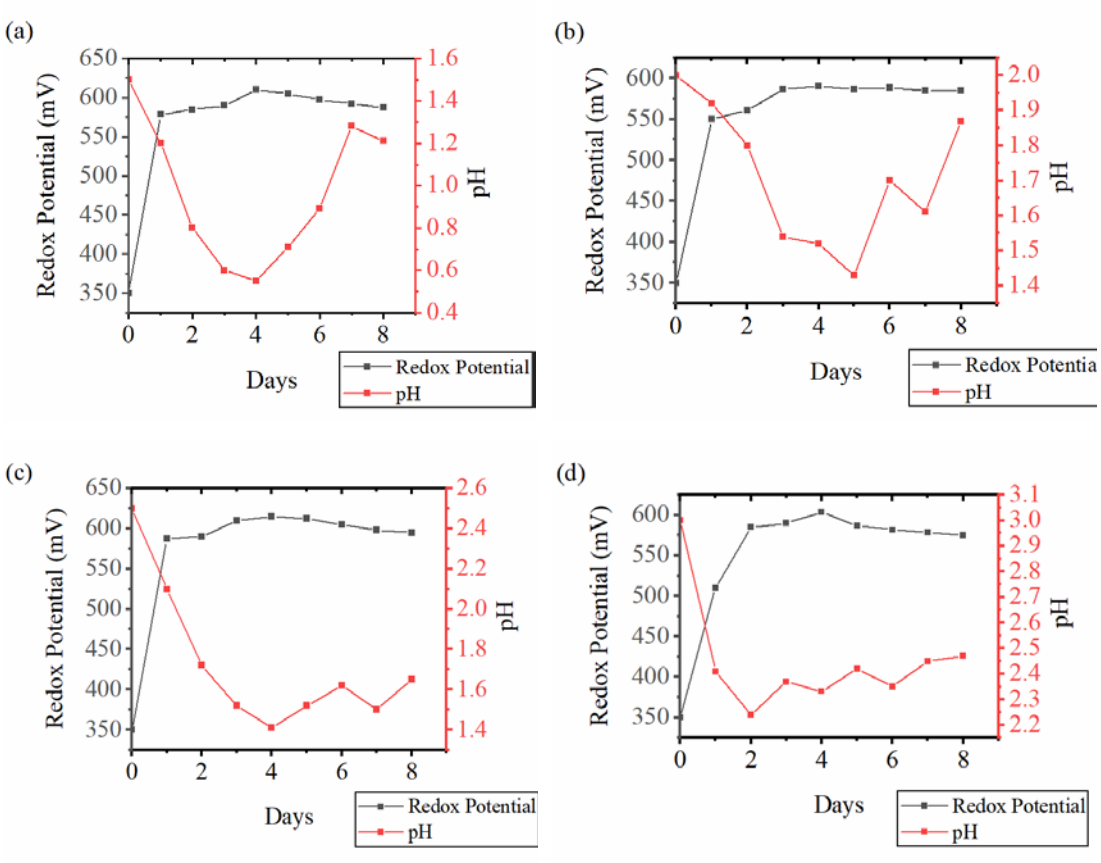


Figure 4. 13. Variation of Redox potential and pH at different initial pH on first experimental investigation (a) 1.5 pH (b) 2 pH (c) 2.5 pH (d) 3 pH.

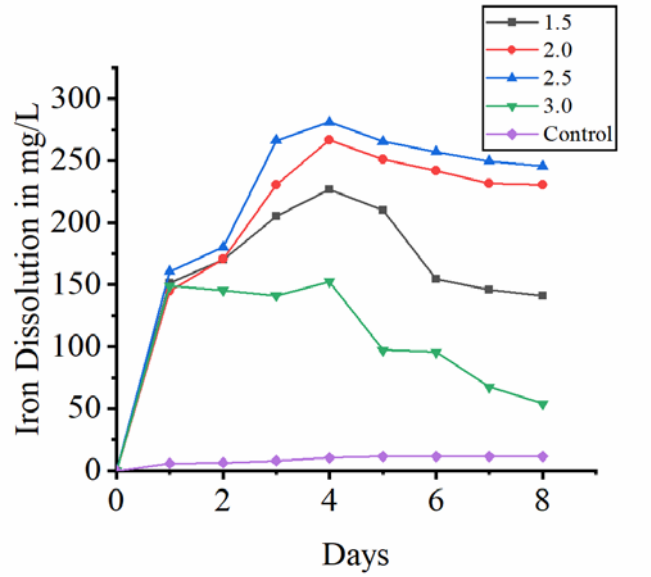


Figure 4. 14. Repeated Bioleaching experiments at different initial pH

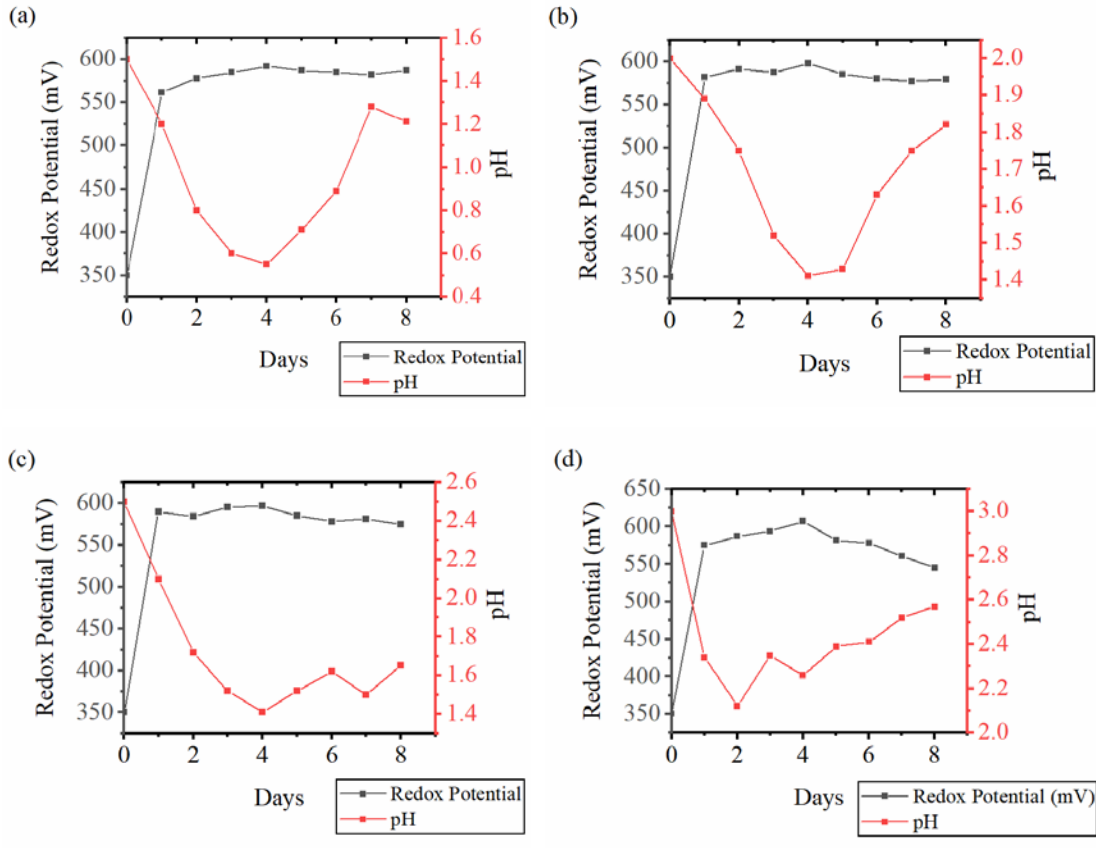


Figure 4. 15. Variation of Redox potential and pH at different pH on repeated experimental investigation (a) 1.5 pH (b) 2 pH (c) 2.5 pH (d) 3 pH.

4.3.4 Effect of Pulp Density:

Figure 4.16 represents the iron dissolution rate at different pulp densities with other parameters constant i.e pH 3.0, shake flask speed 180 RPM, temperature 30 ^{o}C and particle size 150µm. Mousavi and team (2005) found the reduction in copper bioleaching on increase in pulp density from 10 % to 20 % and claims at high pulp density the oxygen supply is limited due to gas liquid mass transfer rendering death of microbial cells (Mousavi et al. 2005a). In the present study at pulp density 5 % maximum iron dissolution of 191.36 mg/L was observed. However on increase in the pulp density to 10 % significant iron dissolution has not been found. Since lateritic soil contains more iron in the form of ferric than ferrous it was the ferric iron load on bacteria hinders the rate of iron dissolution at high pulp density. Pulp density can be kept higher with respect to low ferric iron concentration to obtain maximum leaching (Palencia et al. 1998). This investigation deals with the leaching of iron from the laterite soil which has more ferric iron content than ferrous. Hence maintaining ferric iron content in regard of pulp density does not comes under the scope of this study. Reduction in iron dissolution once again attributed to the oxygen and carbon dioxide limitation at high pulp density (Witne and Phillips 2001).

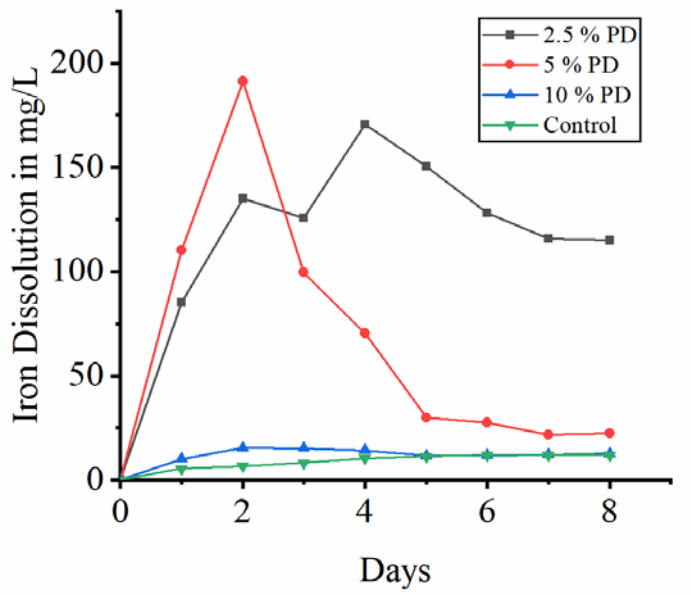


Figure 4. 16. Iron Dissolution on Bioleaching under different pulp density.

There was a sudden increase in the iron dissolution at the pulp density 5 % and reaches to maximum by second day. At the pulp density of 5 % complete oxidation of iron is possible and maximum leaching was observed by the third day of investigation. Iron dissolution at 10 % pulp density indicates the high solid load resulting in insignificant leaching (Nemati and Harrison 2000). Many researchers claim that the higher pulp density limits the oxygen availability for the bacterial activity to prevail. Since the lateritic soil contains high iron content by its composition the study claims that lower pulp density of 5 % is suitable to meet iron requirement of bacteria for its metabolic activity.

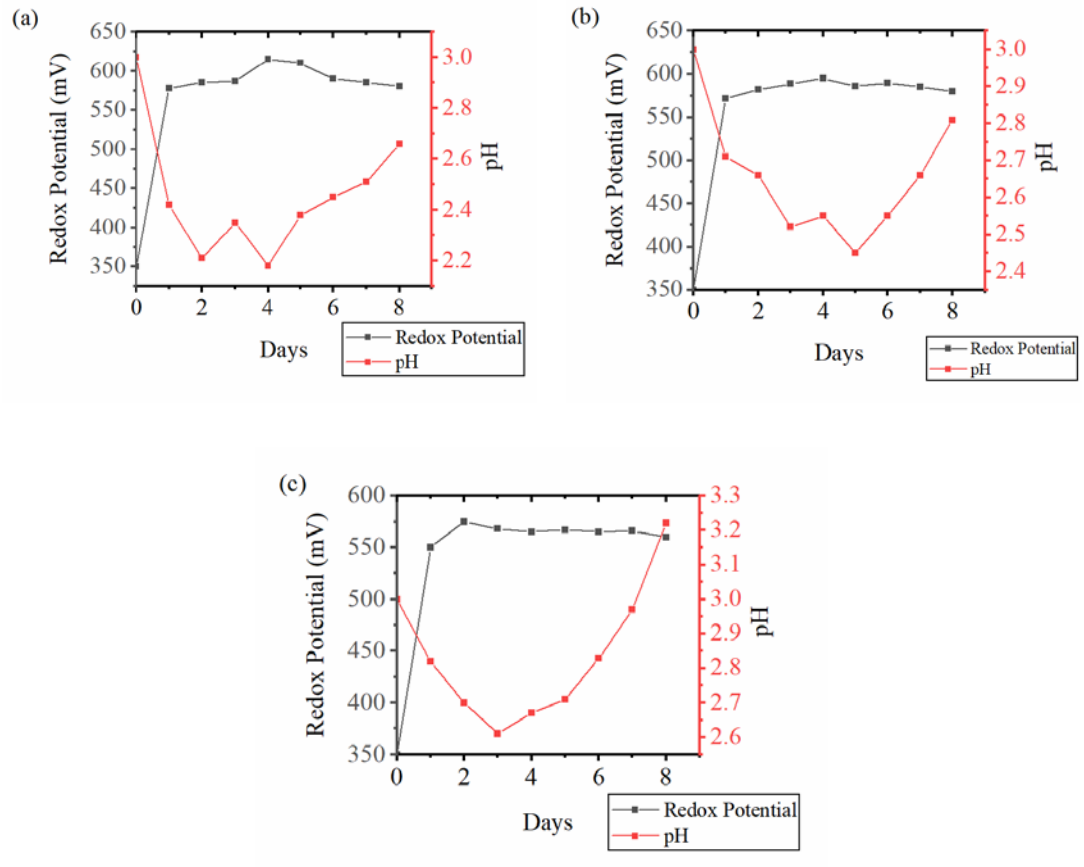


Figure 4. 17. Variation of Redox potential and pH at different pulp density on first experimental investigation (a) 2.5 % PD (b) 5 % PD (c) 10 % PD.

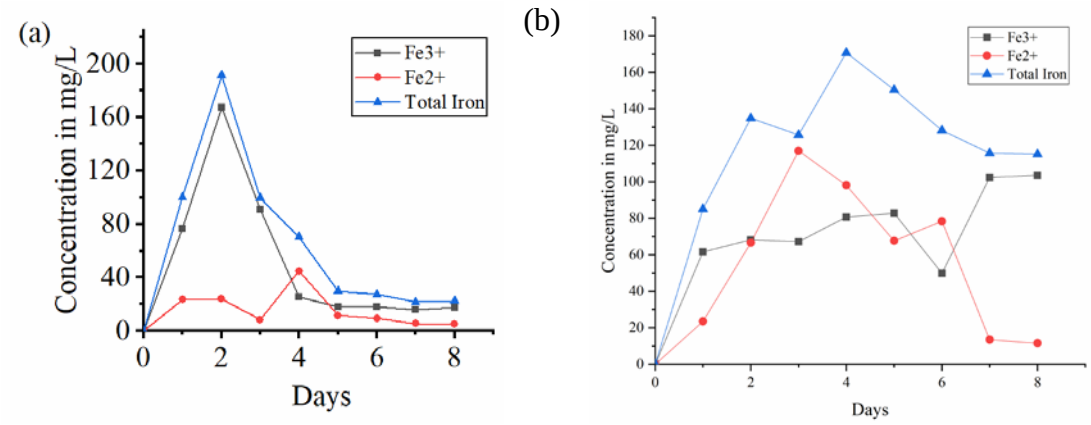


Figure 4. 18. Variation of Ferrous and Ferric Iron during the study at (a) 5 % PD (b) 2.5 % PD.

Figure 4.17 shows the Redox potential and pH variation on different pulp densities and Figure 4.18 demonstrate the variation of ferric and ferrous iron at pulp density 2.5 % and 5% during the study. At 5 % pulp density pH of the solution drops to less than 2.2 by second day in accordance with this the iron dissolution reaches to maximum by second day. Redox potential observed at this condition was more than 600 mV which indicates by the second day the leached iron is oxidized to ferric. In consistence with this result, more ferric iron was observed in the solution indicating bacterial log phase. At 2.5 % pulp density there was a drop in pH by third day and increase in fourth day then again pH drops at a constant rate. The pH lies between 2.3 and slowly increases as the hydrolysis of ferric iron occurs. Redox value at 2.5 % pulp density increases by first day and then the value remain same till the end of the study. At this condition the ferrous iron predominates the ferric iron indicating poor oxidation. This may be due to stationary growth of bacteria. At 10 % the pH drops to 2.6 by third day and thereby shows a rise till the end. Redox potential at this condition remains constant whereas leaching drops to that of control leaching after third day indicating the halt of bacterial activity.

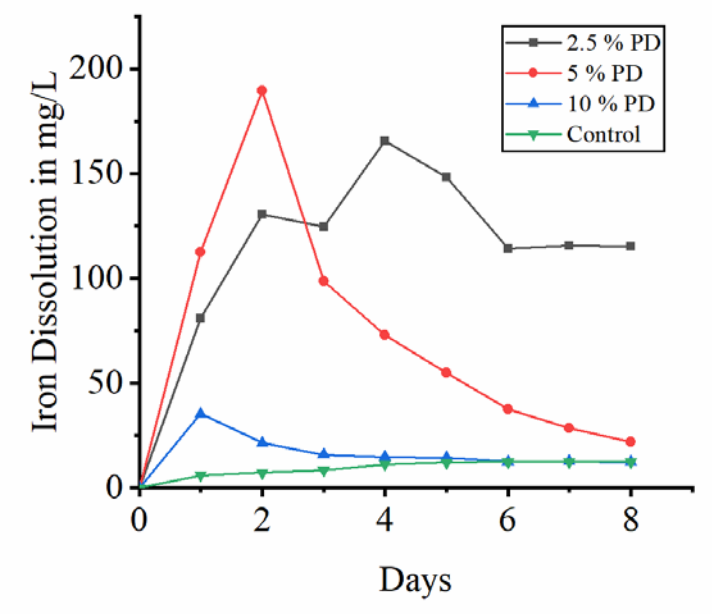
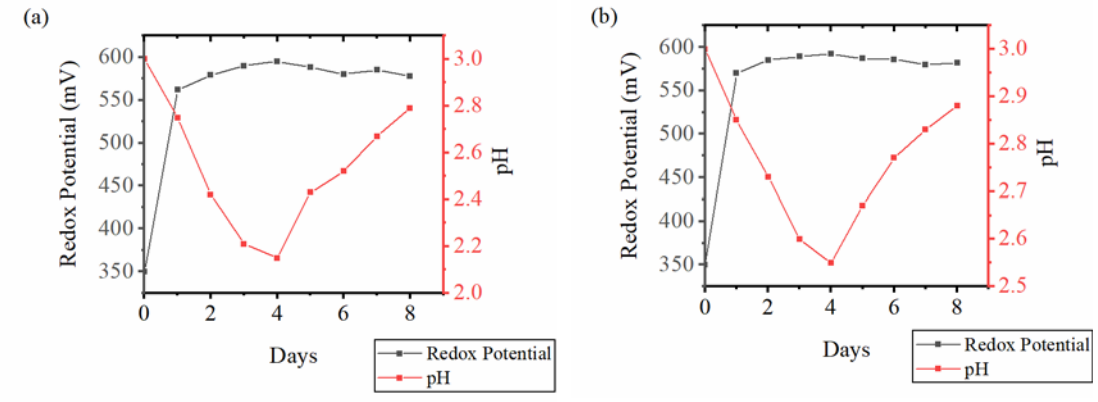


Figure 4. 19. Repeated Bioleaching experiments at different pulp density.

This pulp density is considered as not significant as it indicates high solids loading with respect to lateritic iron leaching. Also it is the gangue material present in the lateritic soil interrupts for bacterial iron leaching at high pulp density.

Repeated experiments conducted to investigate the effect of the pulp density on bioleaching of iron reveals the maximum iron dissolution at 5 % pulp density supporting the previous set of investigation. Figure 4.19 demonstrate the iron dissolution at different pulp density and figure 4.20 shows the variation of redox potential and pH on repeated investigation.



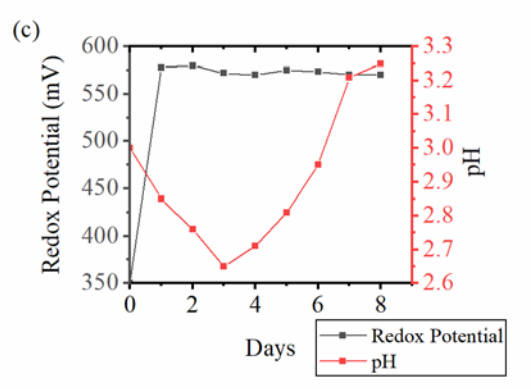


Figure 4. 20. Variation of Redox potential and pH at different pulp density on repeated experimental investigation (a) 2.5 % PD (b) 5 % PD (c) 10 % PD.

4.3.5 Effect of Temperature

Many researchers have been stated that moderate thermophiles are much more efficient in the process of bioleaching compare to mesophilic organisms (Dew D.W et al. 1999; Mousavi et al. 2005b; Qin et al. 2013; Sandstrosm and Petersson 1997). Even though the bacterial strain used here is mesophilic the broad range of temperature to which bacterial adaptation and leaching possibility was evaluated in the mesophilic range (25 ^{o}C – 40 ^{o}C). Figure 4.21 shows the rate of iron dissolution at different temperature with other parameters constant i.e pH 3.0, shake flask speed 180 RPM, pulp density 5% and particle size 150µm. In the present study maximum iron dissolution was observed at 30 ^{o}C indicates the adaptation of bacterial strain to the specific temperature. At higher temperature of 40 ^{o}C the rate of dissolution was reduced drastically and at lower temperature of 25 ^{o}C the rate of dissolution was comparatively less. It is the smaller driving force at low temperature limiting the gas transfer rate making the leaching operation more difficult (Acevedo and Gentina 1989). Gomez et al., (1999) observes the drop in leaching potential of mesophilic bacteria on increase in temperature because of reduction in bacterial activity (Gomez et al. 1999). This is also supported by Karimi and team (2010) as they observed reduction in both iron and copper dissolution rate at 40 ^{o}C. At higher temperature the reduction in bioleaching potential was attributed to bacterial adaptability to particular temperature (Karimi et al. 2010).

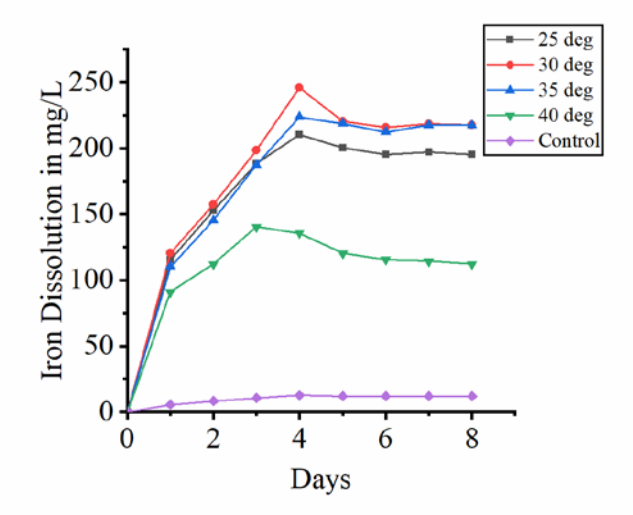


Figure 4. 21. Iron Dissolution on Bioleaching under different temperature.

In the present study the maximum iron dissolution of 245.7 mg/L was observed at 30 ^{o}C temperatures. Figure 4.21 demonstrates the effect of temperature on bioleaching of iron from laterite soil. From graph it can be seen the iron dissolution attains maximum with 30 ^{o}C and 35 ^{o}C. Since the *Acidithiobacillus Ferrrooxidans* strain used here is a mesophilic bacterium, the reason for maximum bioleaching at the observed temperature range is attributed to maximum bacterial activity in this range. At 40 ^{o}C, the iron dissolution observed slowly reaches to maximum by third day and drops at a constant rate. These results are in correspondence with the leaching studies conducted by Karimi and team (2010). The reason attributed to drop in iron dissolution at 40 ^{o}C is the bacterial activity slowly halts with time. Iron dissolution of 211.5 mg/L was observed with the temperature of 25 ^{o}C by the end of the study. In the control experiments without bacteria, the leaching rate was insignificant.

Figure 4.22 demonstrates the variation of redox potential and pH under different temperature during the study. At all the temperature there was a drop in pH by fourth day indicating bacterial oxidation reaches maximum by the fourth day. At temperature 25 ^{o}C the there was a maximum drop initially whereas at 40 ^{o}C the pH drops by second day and then rises to 2.7 by the end of the study. This signifies that the hydrolysis predominates the bacterial iron oxidation giving rise to pH increase at this temperature. Redox potential at 25 ^{o}C reaches the maximum by fourth day whereas redox potential

at 40 ºC the reaches maximum by second day and then the fall in redox potential was observed. Both at 30 ºC and 35 ºC the constant change in redox potential was observed.

On repeated investigation iron dissolution rate was found to be almost same at 30 ºC and 35 ºC (Figure 4.23). The rate of dissolution was 31.3 mg/L per day in the first three days and reaches to a maximum value by the fourth day. The rate of dissolution drops in the next two days and then remains constant till the end of the study. Leaching in the control set is again found to be insignificant on repeated investigation. Redox potential and pH measured during repeated investigation were in favor of first set of experiments (Figure 4.24).

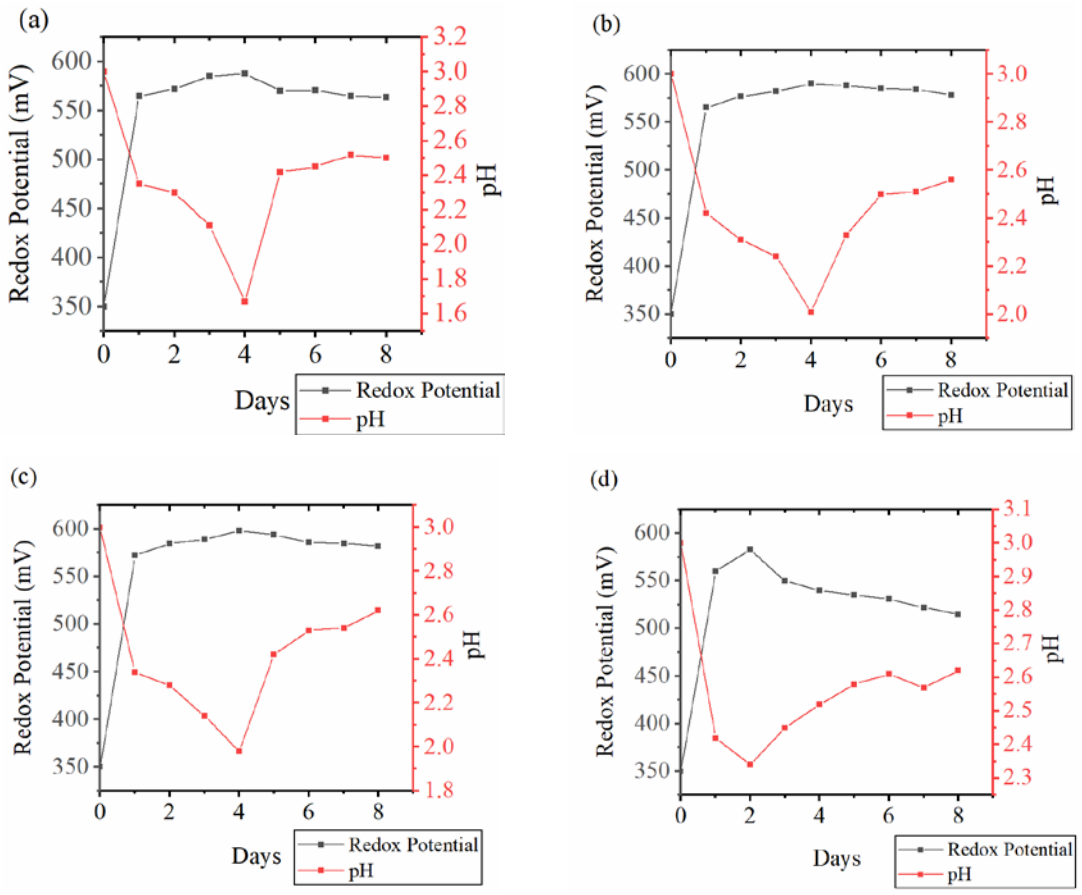


Figure 4. 22. Variation of Redox potential and pH at different temperature on first experimental investigation (a) 25 ºC (b) 30 ºC (c) 35 ºC (d) 40 ºC.

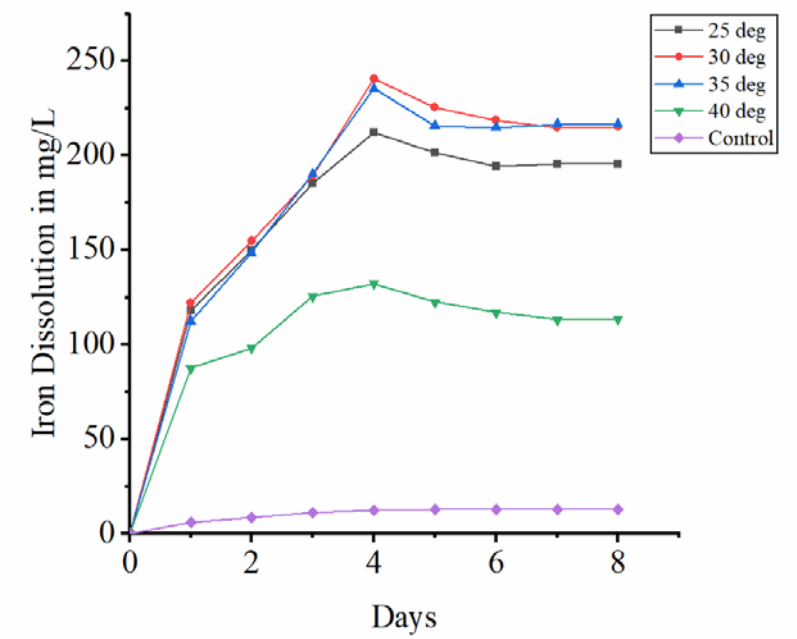


Figure 4. 23. Repeated Bioleaching experiments at different temperature.

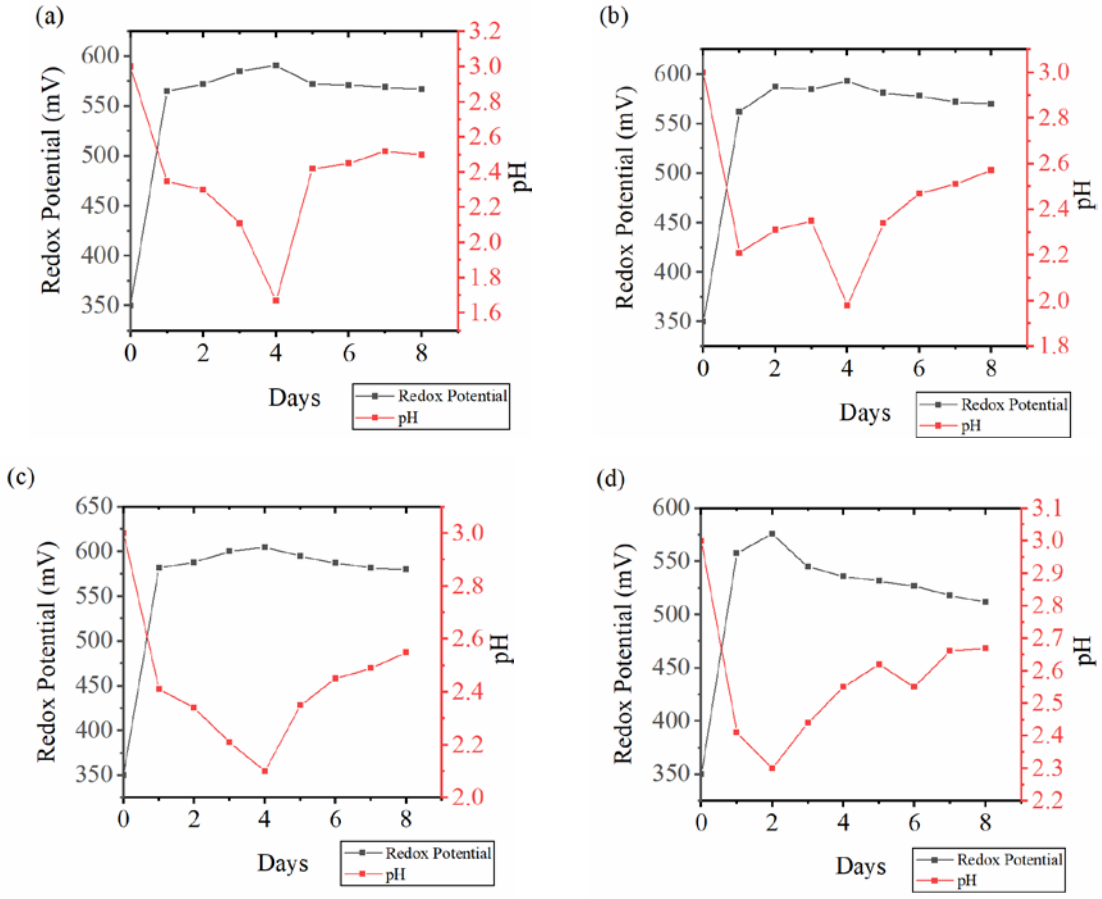


Figure 4. 24. Variation of Redox potential and pH at different pulp density on repeated experimental investigation (a) 25 ºC (b) 30 ºC (c) 35 ºC (d) 40 ºC.

4.3.6 Effect of Particle Size

Particle size plays an important role in bioleaching by regulating bacterial attachment to the surface of mineral ore. Reduction in particle size increases the surface area of the particles thereby increasing the rate of metal dissolution (Blancarte-Zurita et al. 1986; Devasia et al. 1993; Torma et al. 1972). Smaller the size more the iron dissolution occurred. This may be because of smaller particles (less than 100µm) facilitates the uniform slurry and increased active sites of mineral (Acevedo and Gentina 1989). Lower particle size also enhances the bacterial attachment to laterite resulting in more iron dissolution (Qiu et al. 2006). However in the present study after first four days, the rate of dissolution is reduced because of ferric precipitation. The initial pH drop at first four days in the setup with particle size less than 75µm and high redox potential confirms the high biooxidation rate at lesser particle size and hence more dissolution of iron.

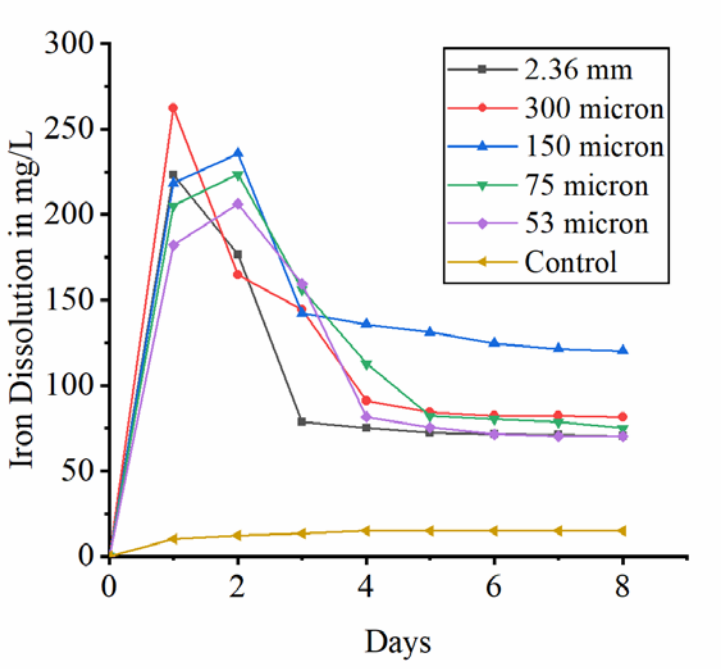
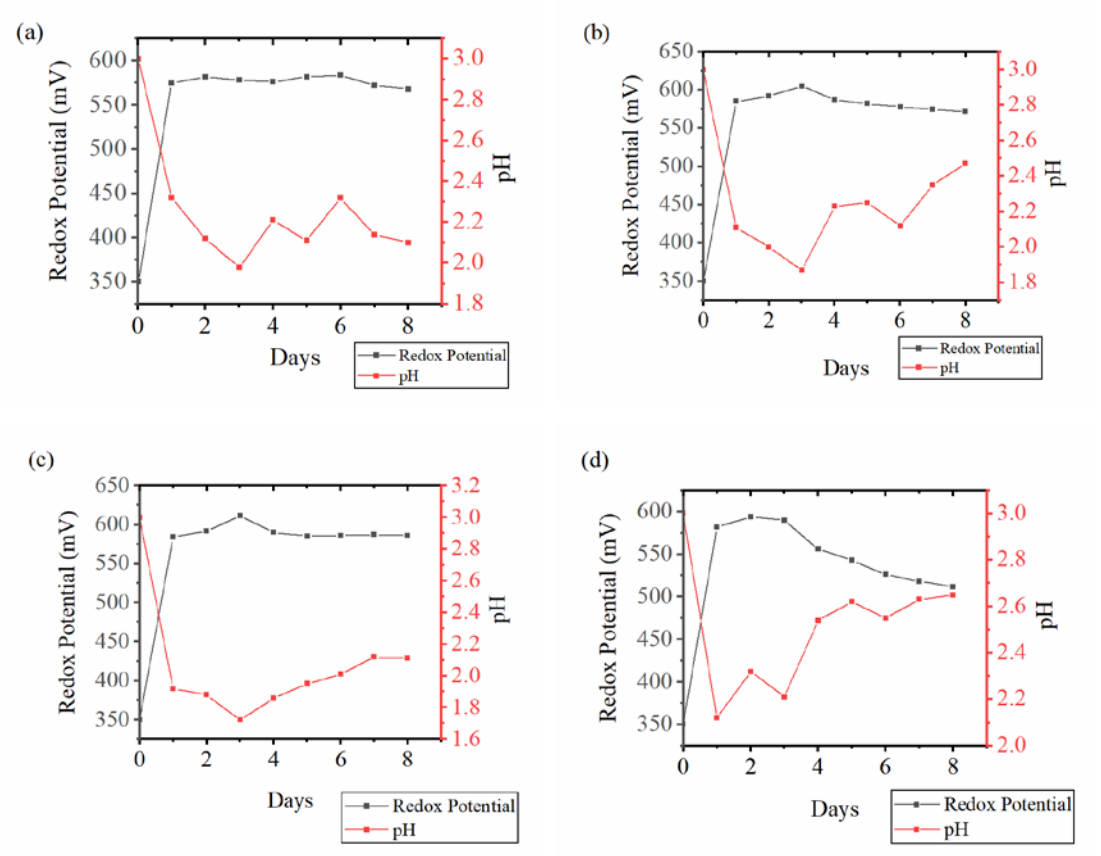


Figure 4. 25. Iron Dissolution on Bioleaching at different particle size.

Maximum iron dissolution of 241.6 mg/L was observed with the finer particle size of less than 75 micron. Figure 4.25 demonstrates the effect of different particle size on the bioleaching of iron with other parameters constant i.e pH 3.0, shake flask speed 180 RPM, temperature 30 ^{o}C and pulp density 5%. Observing on graph it is shown that there

is a drastic increase in the iron leaching with particle size less than 75 micron. Reaching to the maximum rate by fourth day it has been observed with the sudden drop in leaching rate. In contrast to this leaching with other particle size reaches its maximum and constantly shows the decrease at the end of the study. It is accomplished that particle with size less than 75 micron favors the bacterial attachment to mineral providing more surface area. Thus in the initial days the leaching was found to be maximum and after fourth day the leached iron on oxidizing to ferric forms precipitation which covers the mineral surface hindering the bacterial attachment thereby lowering the leaching rate.



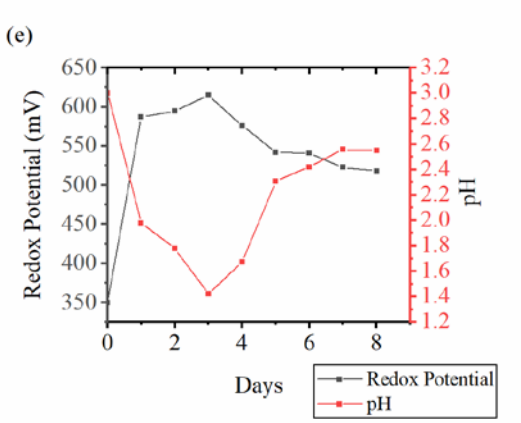


Figure 4. 26. Variation of Redox potential and pH at different particle size on first experimental investigation (a) 2.36 mm (b) 300 micron (c) 150 micron (d) 75 micron (e) 53 micron.

During this study initial pH drops to less than 2 favoring the ferrous oxidation and ferric precipitation is indicated by the rise in pH. On the other hand the particle size of 75 micron, 170 micron and 300 micron shows the constant leaching rate which starts to drop after fourth day indicating the lesser bacterial activity. In case of particle size of 2.36 mm it is observed that there was no significant iron leaching since the particle size does not favors the bacterial attachment.

Figure 4.26 demonstrates the variation of redox potential and pH under different temperature during the study. With particle size 150 micron and 53 micron the redox potential reaches its maximum by third day of the study indicating maximum biooxidation with the corresponding pH drop to less than 1.5. In consistent to this data iron dissolution rate for particle size 150 micron and 53 micron reaches its maximum by third day of the study. However the maximum iron dissolution was observed with 300 microns within one day and the redox potential with this particle size shows constant increase and drop after fourth day of the study.

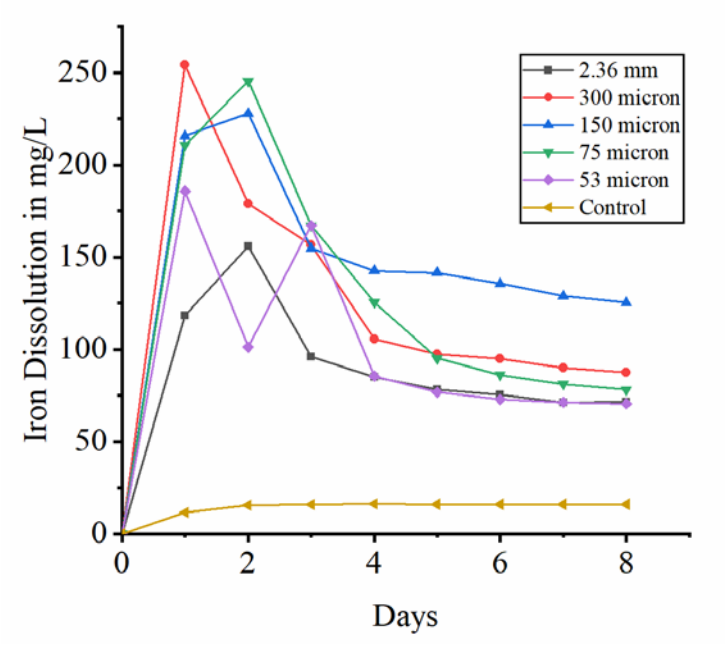
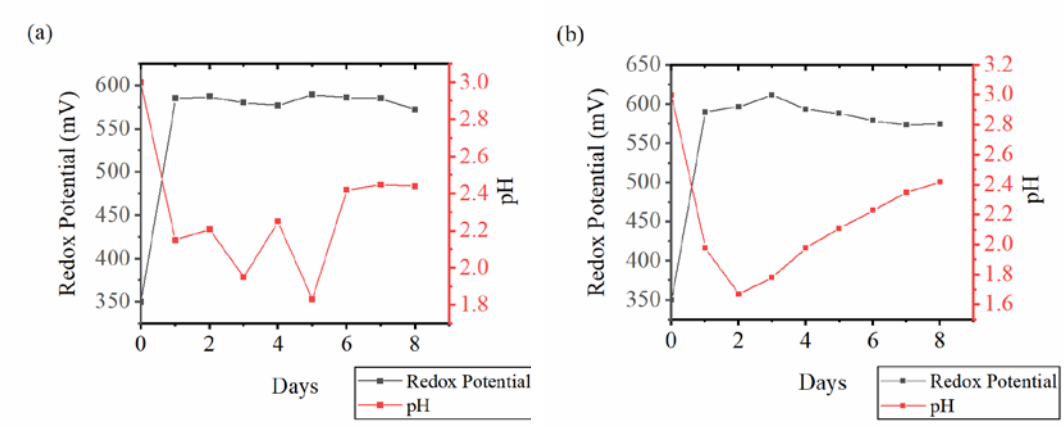


Figure 4. 27. Repeated Bioleaching experiments on different particle size.



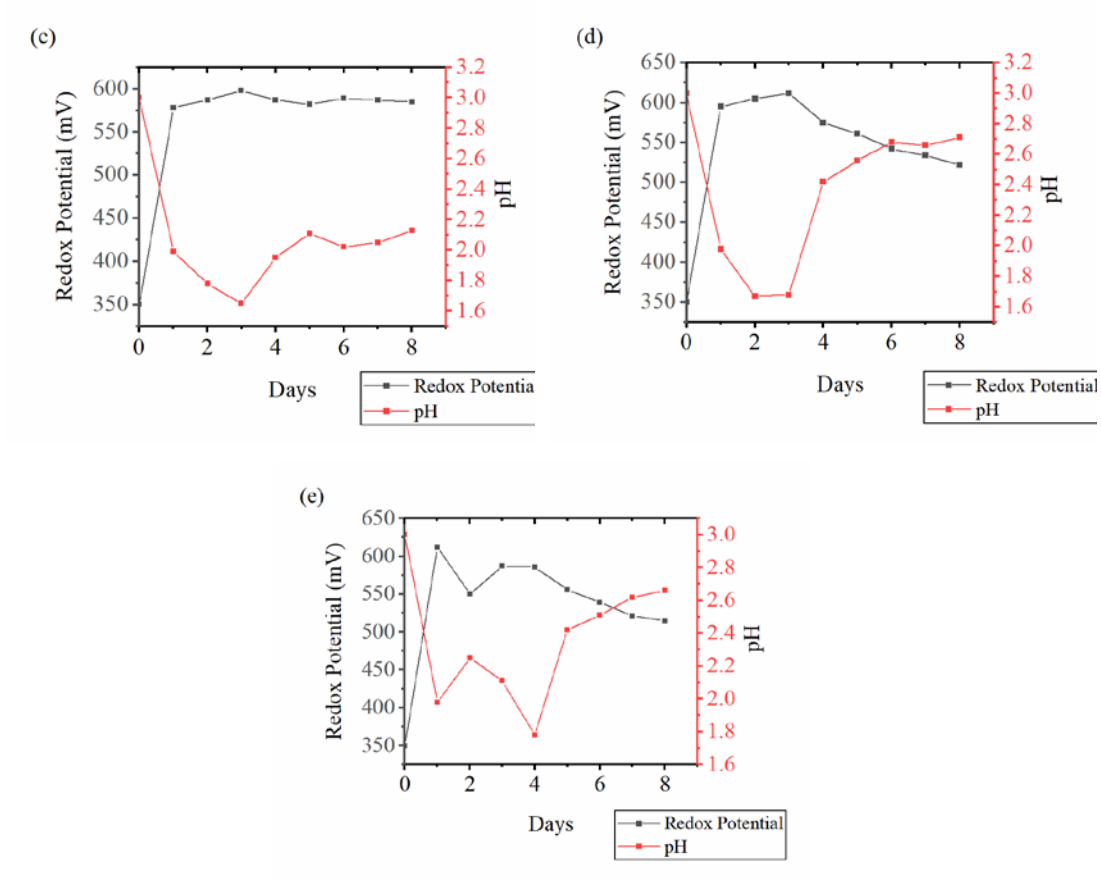


Figure 4. 28. Variation of Redox potential and pH at different particle size on repeated experimental investigation (a) 2.36 mm (b) 300 micron (c) 150 micron (d) 75 micron (e) 53 micron.

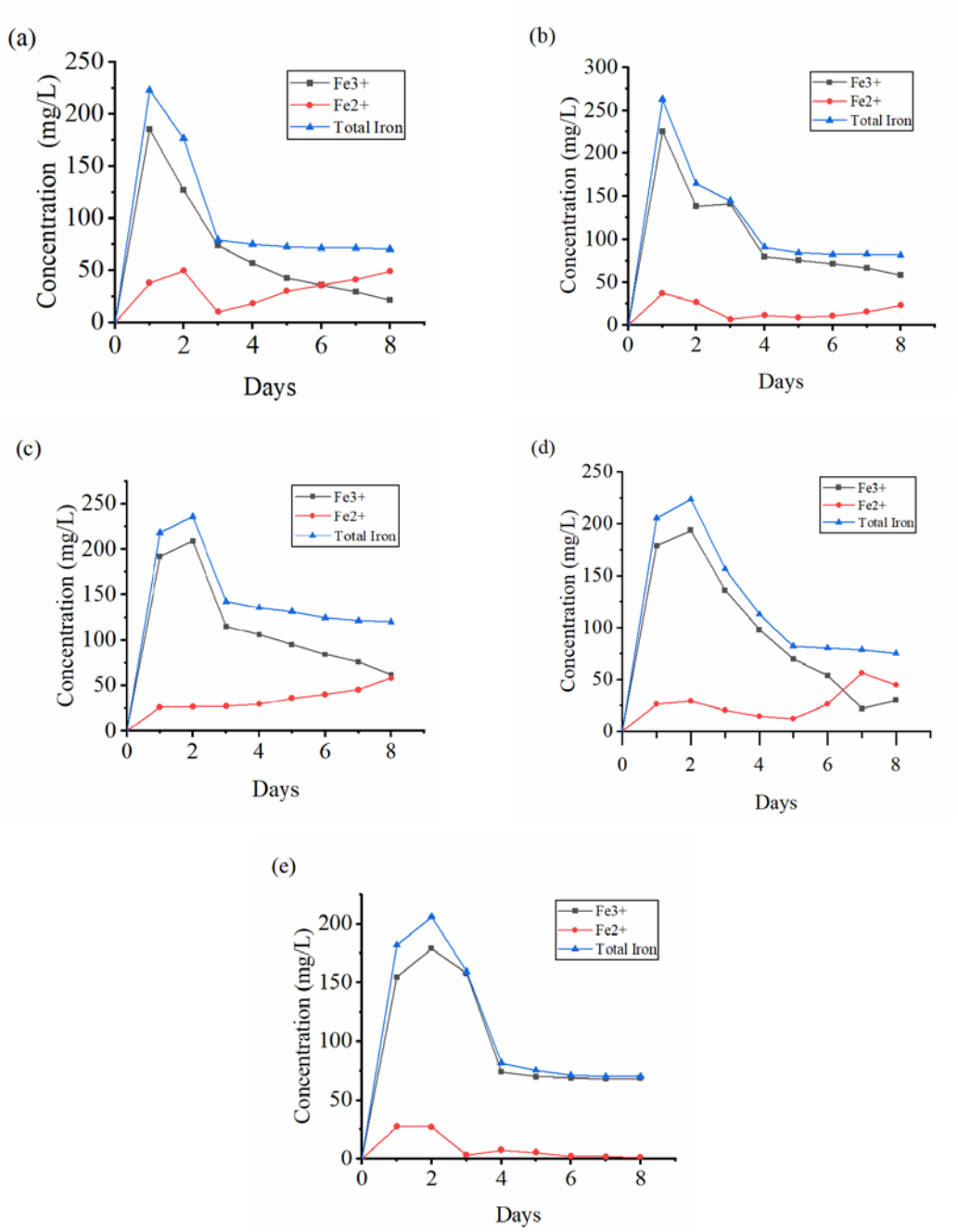


Figure 4. 29. Variation of Ferrous and Ferric Iron during the study at (a) 2.36 mm (b) 300 micron (c) 150 micron (d) 75 micron (e) 53 micron.

Iron dissolution on repeated investigation shows sudden drop in iron dissolution with particle size of 53 micron which is attributed to ferric precipitation with the smaller size of particles (Figure 4.27). Variation of Redox potentian and pH on repeated experiments are graphically presented in the figure 4.28.

Ferrous and ferric were measured separately to trace the effect of particle size on iron dissolution rate (Figure 4.29). With the particle size 300 micron and 53 micron ferrous iron concentration is almost zero by the third day with corresponding ferric concentration being maximum. The concentration of ferrous increases gradually with other particle size whereas with 53 micron it shows no increase. The decrease in ferrous iron is attributed to ferrous oxidation while the decrease in ferric concentration is due to precipitation formation. The study claims that with lesser particle size 53 microns the ferric precipitates formed forms the layer around fine particles thereby hindering the bacterial contact with lateritic particles hence the continuous drop in both ferric and ferrous iron can be observed.

4.3.7 Effect of Sulfate supplement on Bioleaching of iron

There was no considerable enhancement in the rate of bioleaching of iron on additional sulfate supply of about 28 g/L. Figure 4.30 demonstrates the sulfate concentration during bioleaching of iron from laterite soil. It has been observed that there was a decrease in sulfate concentration provided with all pulp density by the first day. In consistent to this the bacterial oxidation was found to be maximum both with sulfate and without sulfate supplement. Sulfate concentration in the solution increases by the fourth day gradually indicating no sulfate consumption more. This might be due to inhibition of bacterial activity. The study claims that sulfate supplement does not cause any significant increase in the rate of leaching of iron from laterite soil. It is the gangue material present in the laterite causes the inhibition in bacterial activity.

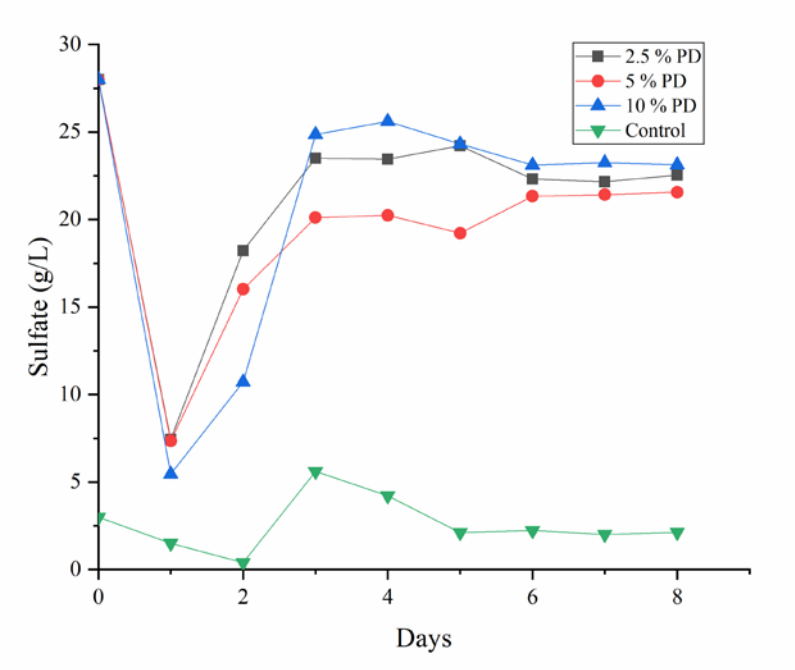


Figure 4. 30. Variation of sulfate during bioleaching at different pulp densities.

4.3.7 Characterization of Bioleached Lateritic Soil:

Bioleached lateritic soil was examined for its mineralogical composition and also for morphological appearance.

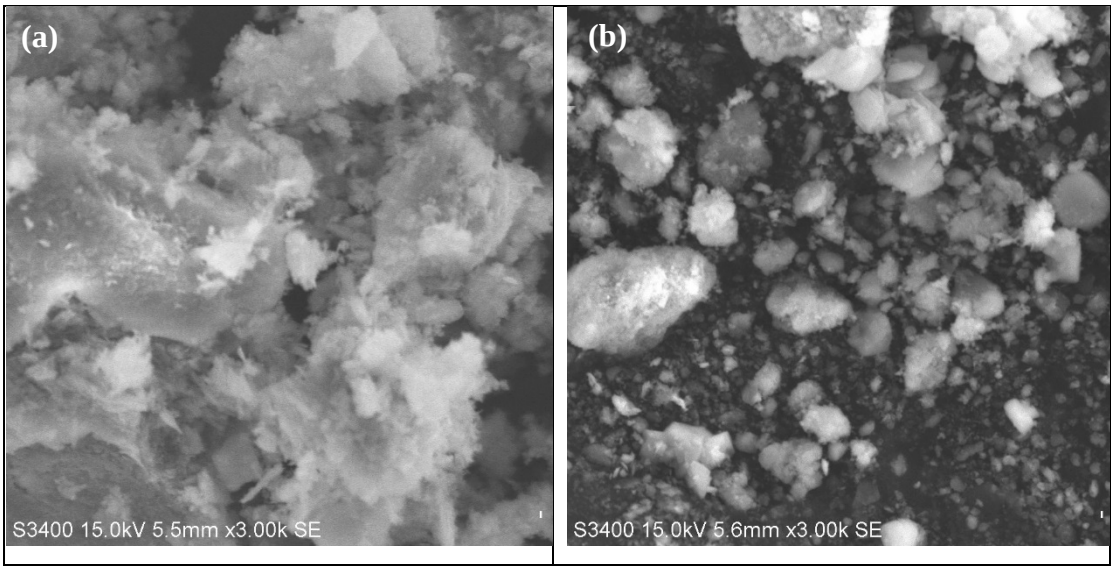


Figure 4. 31. SEM images of Lateritic Soil a) Fresh b) Bioleached

On bioleaching the amorphous and fibrous like structure of fresh lateritic soil was completely changed under the influence of bacterial action. It is observed that there is a shift in peak after leaching indicating the change in the mineral composition of the study soil with the presence of jarosite (PDF No. 01-071-1777) and Iron oxide hydrated (PDF No. 00-001-0662) at 2Ɵ 46.72, 62.40, 68.42 (figure 4.32) . SEM images of fresh and 8 days old bioleached lateritic soil is presented in which bacterial attachment has not been observed due to jarosite precipitation by 8th day (Figure 4.31). Excess ferric iron concentration in the present study has negative effect on the iron dissolution rate. EDS spectrum shows the peak representing iron, aluminium, sulphur, cobalt, nickel (Figure 4.33). The sulphur in the spectrum might be due to sulphate supplement for bacterial activities to accur.

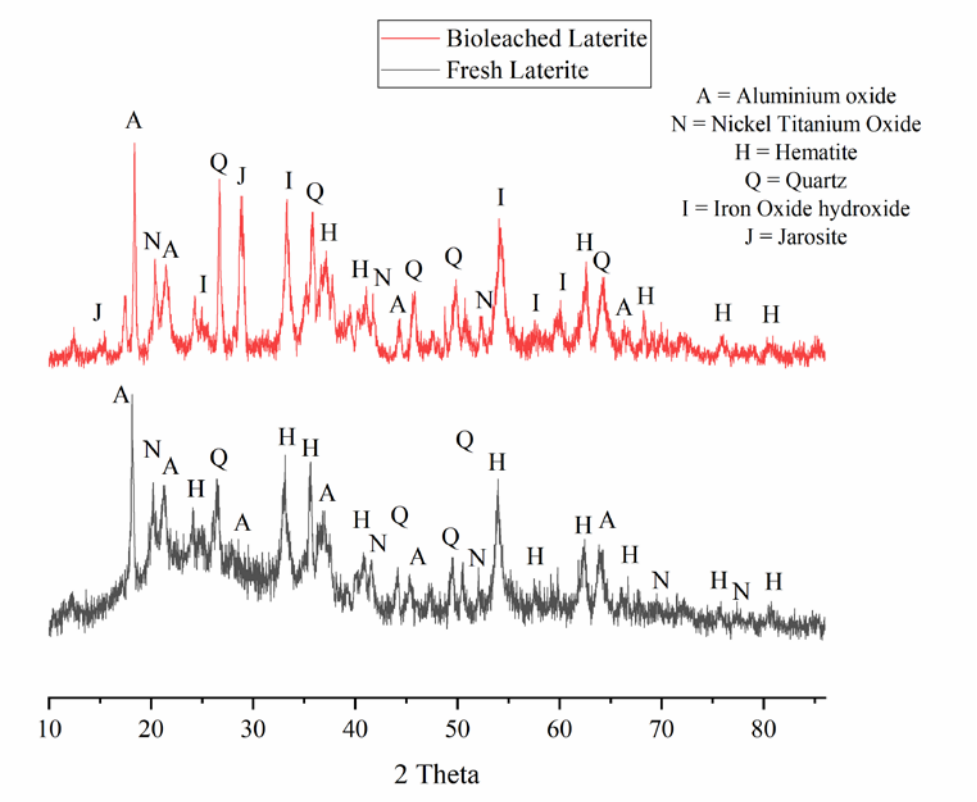


Figure 4. 32. XRD pattern of study soil before and after leaching

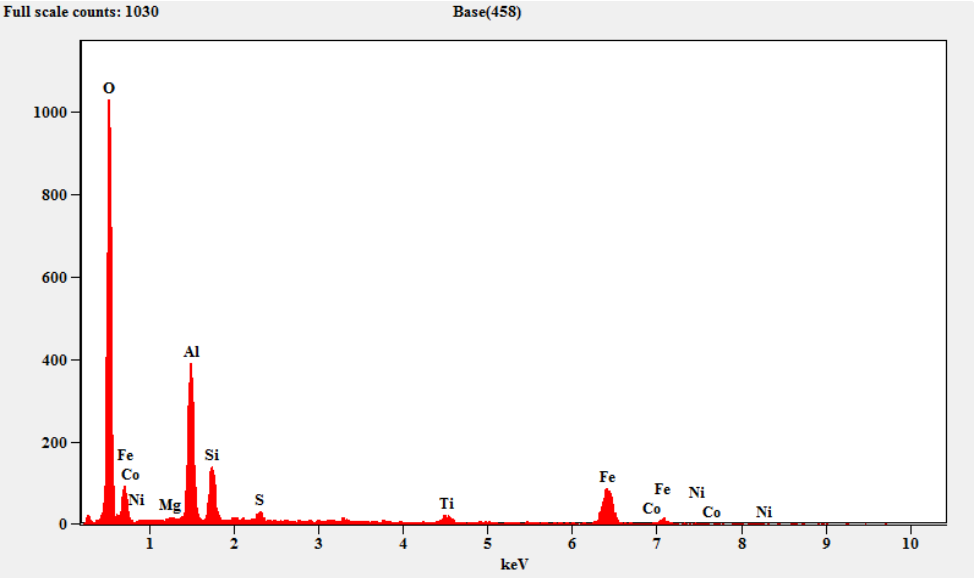


Figure 4. 33. EDS of the study soil after leaching

4.4 FENTON'S OXIDATION OF SELECTIVE HERBICIDES:

4.4.1 Catalytic Role of Biosynthetic Jarosite in Fenton's degradation Selective Herbicides

4.4.1.1 Fenton's Degradation of Ametryn

Degradation of ametryn by jarosite catalyzed heterogeneous Fenton like process was investigated. Initially no removal of ametryn was observed without the addition of H_2O_2 in solution containing ametryn and jarosite. The degradation however starts slowly on the addition of H_2O_2 initiating the reaction. High removal efficiency upto 84.90 % was found with jarosite dosage of 0.5 g/L and H_2O_2 dosage of 100 mg/L at pH 3.0 and temperature 30^{o}C. The COD removal was 51.6 % in first 120 min. This signifies the better oxidation rate. Studies on the influence of H_2O_2 in pollutant removal at different jarosite concentration revealed that the increase of H_2O_2 from 100mg/L to 200mg/L, 500mg/L and 1000mg/L did not show significant change. And confirms further increase in dosage than 100mg/L did not led to shortening of induction period (Yan et al., 2017). During the reaction, there was slight increase in pH 3.0 to 4.21. Fenton's reaction is active in acidic pH as the condition favors the oxidation and production of OH radicals. If the pH turns basic the hydrated ferrous ions gets transformed into colloidal ferric species i.e ferric hydroxyl complexes which results in drop of degradation efficiency

(Burbano et al., 2005; Kang, Y. and Hwang 2000; Khan et al., 2009). The lower pH observed during the treatment favored the catalytic degradation of ametryn producing high quantity OH radicals. Previous studies have shown that even though when pH is greater than point of zero charge biosynthesized jarosite consumes hydroxyl radicals resulting in pH drop, the hydroxyl bridges of jarosite re-polymerize to further drop in pH (Yan et al., 2017). The increase in the rate of ametryn degradation on increase in jarosite load from 0.1g/L to 0.5g/L indicates the high iron concentration favors the Fenton's oxidation as it helps in H_2O_2 decomposition to produce more OH radicals by the acceleration of active sites on the catalyst. In the present study, 84.90 % of ametryn degradation was observed. The kinetic rate increased nearly by 1.2 times by the increase in catalyst loading from 0.1 g/L to 0.5 g/L however increase in jarosite loading more than this dosage did not show any significant changes. Figure 4.34 graphically represents the effect of H_2O_2 on the oxidation process and figure 4.35 represents the variation in ferric and total iron contents during the process. Kinetic studies show that pseudo-first order rate kinetic model fits the process (Chen et al., 2017). Figure 4.36 shows the kinetic fit.

Table 4.04 shows the intermediates formed during the Fenton's oxidation of ametryn using biogenic jarosite. Ametryn on this degradation process follows alkylic oxidation and hydroxylation pathways. With two dealkylation intermediates 2 – acetamido – 4 (iso propylamino) – 6 – (methylthio) – s triazine (SDIT) and 2 – amino – 4 (ethylamino) – 6 – (methylthio) – s triazine (SEAT) resulting from the oxidation of alkyl group in the lateral chain to form acetamide-aldehyde compounds and one hydroxylation intermediate 2 – ethylamino – 4 hydroxy - 6 (isopropylamino) – s triazine (OIET) identified. The OH radicals produced attacks the lateral chain of heterocycle in the dechlorination hydroxylation process to form OIET. Chen et al. 2018 claims deisopropylation of ametryn gives SEAT with m/z value 186.08 identified in the present study. The m/z value of 198.13 in the present study confirms the formation of dechlorinated hydroxylated intermediate 2 – ethylamino – 4 hydroxy - 6 (isopropylamino) – s triazine (OIET) from the initial hydroxylation of ametryn which later follows dealkylation pathway. Dechlorinated product undergo dealkylation process to form dechloro - dealkylated biproducts 2 – hydroxyl-4-isopropylamino-6-

diamino triazine (OIAT) and 2-hydroxly-4,6-diamino triazine (OAAT) with m/z value 170.10 and 128.05 respectively; OAAT being the final product of Fenton's oxidation. The intermediate compounds formed in this study within 20 minutes of treatment were in consistent with Chen et al., and Oliveira et al. Strong electron donating effect of methylthio group reliable for hydroxyl radical attack makes the ametryn more susceptible for Fenton's oxidation compare to the other herbicides (Chen et al., 2017; Oliveira et al., 2019).

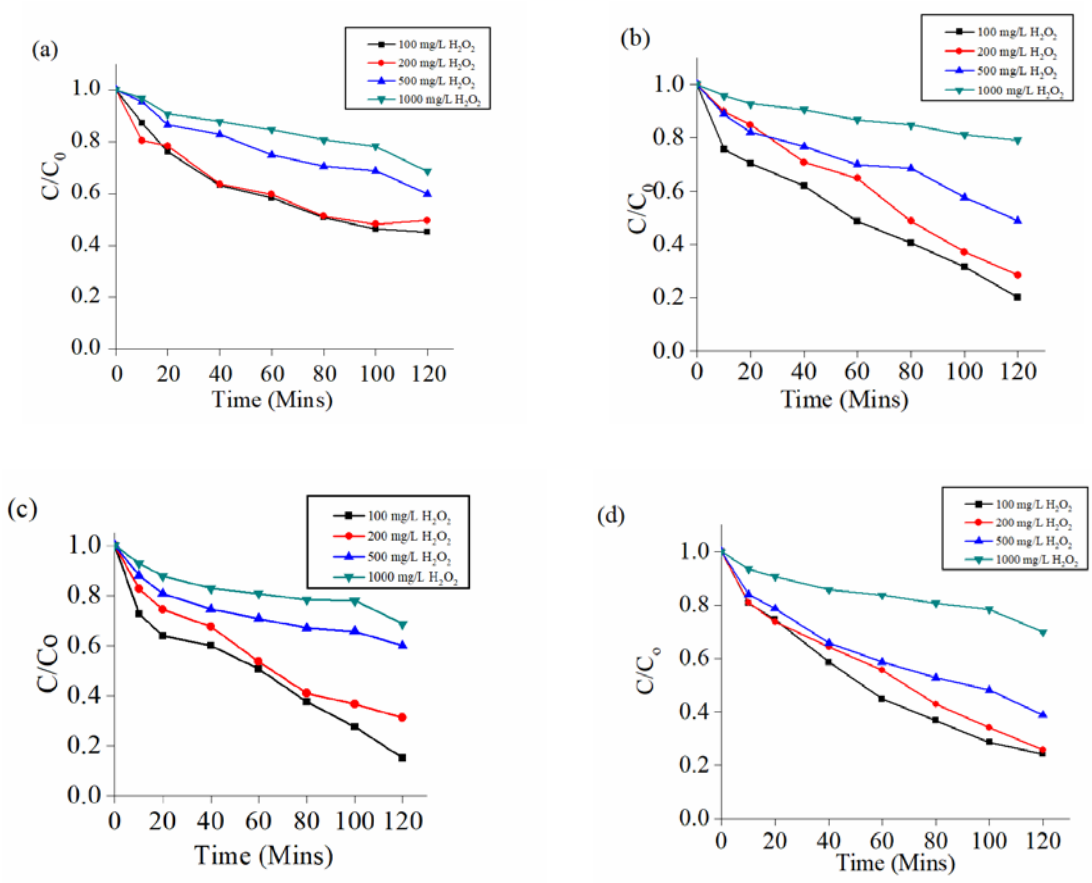


Figure 4. 34. Effect of H_2O_2 concentration on the process at different jarosite loading a) 0.1g/L jarosite b) 0.2g/L jarosite c) 0.5g/L jarosite and d) 1.0g/L jarosite. Ametryn degradation

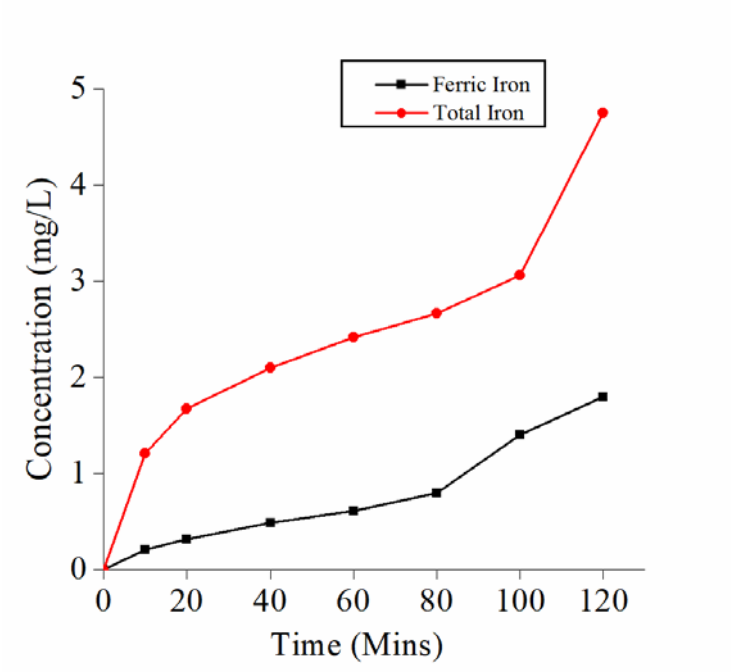


Figure 4. 35. Variation of Ferric iron and total iron during the Fenton's process Ametryn degradation

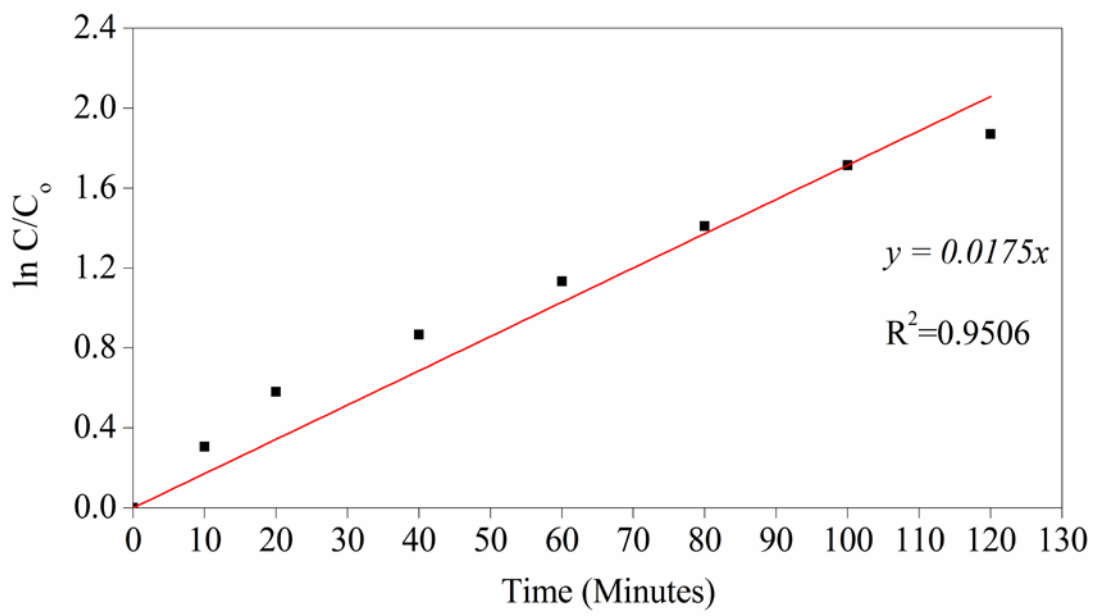


Figure 4. 36. First order kinetic model fit ln C/C$_o$ versus time Ametryn degradation

Table 4. 04. Intermediates formed during the Fenton's oxidation of Ametryn in the present study

m/z	Chemical formule	Identified by
212.1405	$C_8H_{13}N_5O_2$	de Olievera et al., 2018
200.0950	$C_7H_{13}N_5S$	de Olievera et al., 2018; Chen et al., 2016
198.1343	$C_8H_{15}N_5O$	de Olievera et al., 2018; Chen et al., 2016
186.080	$C_6H_{11}N_5S$	de Olievera et al., 2018; Chen et al., 2016
170.1022	$C_5H_7N_5O_2$	de Olievera et al., 2018
158.0488	$C_4H_7N_5S$	de Olievera et al., 2018; Chen et al., 2016
128.0541	$C_3H_5N_5O$	de Olievera et al., 2018

4.4.1.2 Fenton's Degradation of Dicamba:

Oxidative degradation of dicamba by jarosite catalyzed Fenton's process was investigated. Initiation of oxidation on the addition of H_2O_2 reveals us the degradation of dicamba follows the Fenton's reaction. Removal efficiency up to 91.29 % was found with jarosite load of 0.5g/L and H_2O_2 dosage of 1000 mg/L at pH 3. Reduction of COD value from 320 mg/L to 107.56 mg/L within 120 min of reaction indicates the better oxidation rate. During Fenton's oxidation hydrated ferrous ions gets transforms into colloidal ferric species forming the ferric hydroxyl complexes thereby reducing the degradation efficiency at basic pH. Hence it is proven that the Fenton's oxidation process is active in acidic pH (Burbano et al. 2005; Kang, Y. and Hwang 2000; Khan et al. 2009). In the present study there are no notable changes in pH value maintained during the process. However the slight changes shifts to acidic favoring the Fenton's process. The lower pH observed during the treatment favored the catalytic degradation of dicamba producing high quantity OH radicals. Effect of catalyst loading was studied by increasing jarosite loading in the range of 0.1 g/L to 1 g/L. During the study, the degradation of dicamba increased with the increase in jarosite load. This indicates the jarosite has a role of catalyst in the process by decomposing hydrogen peroxide into

hydroxyl radicals thereby accelerating the active sites on the catalyst. Increase in the catalyst load from 0.1 g/L to 0.5 g/L leads to increase in the pollutant degradation by 43.6 % however increase in dosage more than this does not show any efficient degradation indicating 0.5 g/L of jarosite as optimum dosage. Figure 4.37 shows the effect of H_2O_2 on the dicamba degradation with 0.5 g/L of jarosite dosage. Plot shows the increase in degradation with increased H_2O_2 dosage from 100 mg/L to 1000 mg/L. This reveals that the influence of H_2O_2 on the process, as the dosage increases more hydroxyl radicals liberated involves in the oxidative degradation of dicamba (Yan et al. 2017). Ferric iron leached out from the mineral initially reacts with H_2O_2 to get reduced to ferrous ions. At this stage the hydroxyl radicals formed attack the aromatic ring to remove the chlorine resulting in the formation of hydroxylated aromatic products thereby initiating the degradation process (Drzewicz et al. 2005; Egusa et al. 2011). It is the presence of electron withdrawing groups of dicamba at ortho and para positions to the chlorine atoms bring out the dehalogenation reaction (Maya-treviño et al. 2014). The electronegative properties of carboxyl groups present in the dicamba weakens carbon-chloride bond making it susceptible for the degradation (Ghauch 2001). Due to this weak bond the elimination of chlorine occurs successively from the beginning of the reaction. Figure 4.39 shows the variation of chloride measured at different time intervals during the reaction. At 40 mins the observed chloride indicates the chlorine elimination starts from the beginning. Drzewicz and his colleagues (2005) observes that at complete degradation of dicamba the 50% of chlorine associated is converted into inorganic chlorine leaving behind the rest with organic compound only. Degradation of dicamba reached its maximum in 80 mins whereas COD value at the time was 209.31 mg/L with mineralization efficiency 43.59 % indicating the degradation of dicamba occurs prior to maximum reduction in COD (Huston and Pignatello 1999). Huston and Pignatello (1999) reported the complete degradation of dicamba within 120 min by photo fenton process (Huston and Pignatello 1999). The variation of total iron during the process with 0.5g/L jarosite at different H_2O_2 dosage is shown in the figure 4.38. As seen in the plot there is no significant iron leached out initially and the total iron leached into the system was 3.81 mg/L with 0.5 g/L of jarosite and 1000 mg/L of H_2O_2 dosage. In spite of this the reduction in measured dicamba

concentration occurs within the 20 mins of process which indicates the reduction in dicamba may be due to adsorption on the mineral surface (Meng et al. 2017). This again reveals the Fenton's reaction is not homogeneous but the Fenton's oxidation of dicamba with jarosite as catalyst follows heterogeneous reaction. Figure 4.40 shows the kinetic fit. Kinetic studies show that pseudo-first order rate kinetic model fits the process (Chen et al. 2017).

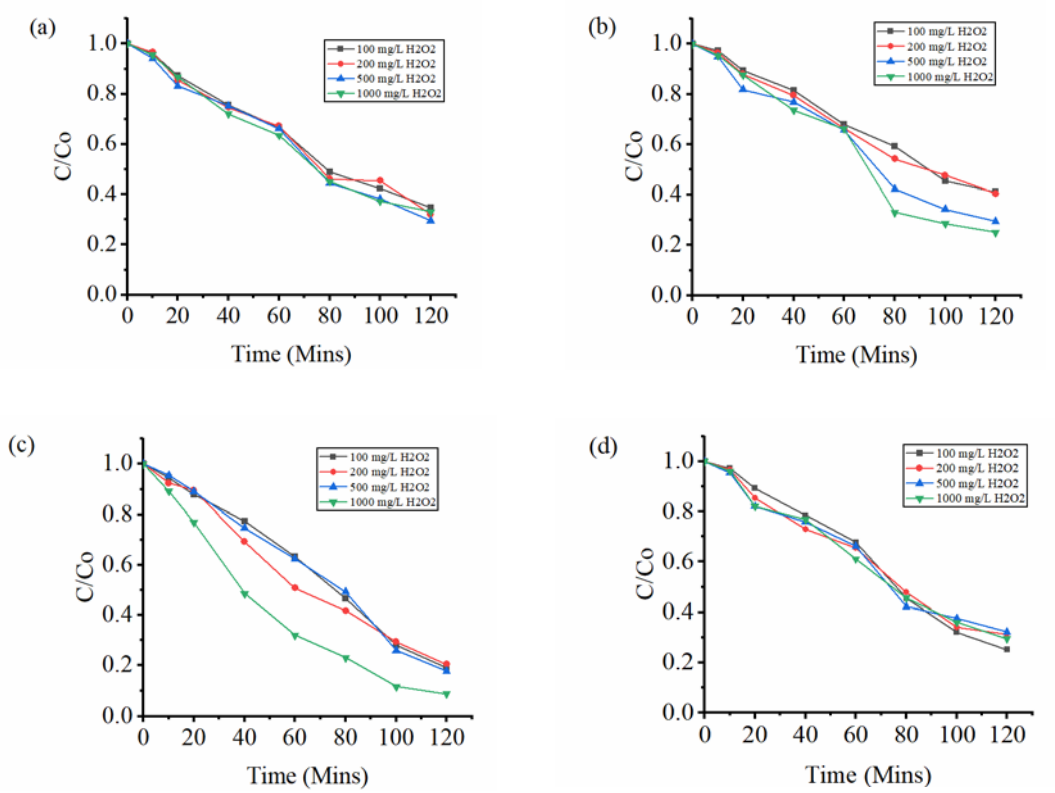


Figure 4. 37. Effect of H_2O_2 concentration on the process at different jarosite loading a) 0.1g/L jarosite b) 0.2g/L jarosite c) 0.5g/L jarosite and d) 1.0g/L jarosite for Dicamba degradation.

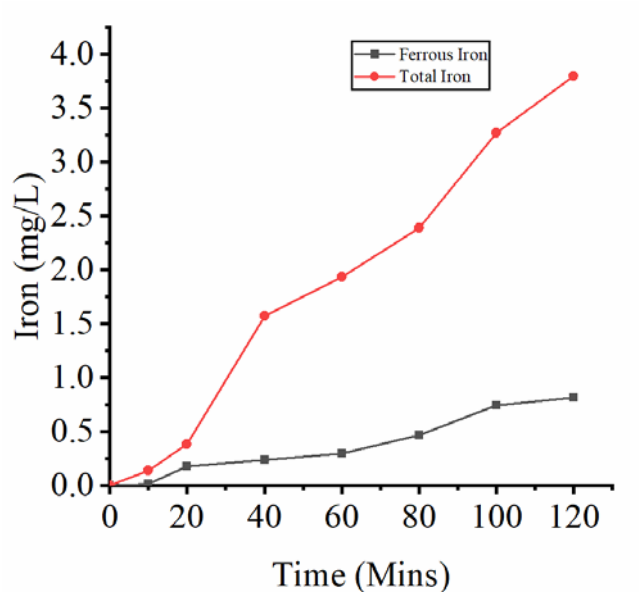


Figure 4. 38. Variation of Total iron and ferrous iron at 0.5 g/L of Jarosite loading and different H_2O_2 dosage during the process for Dicamba degradation.

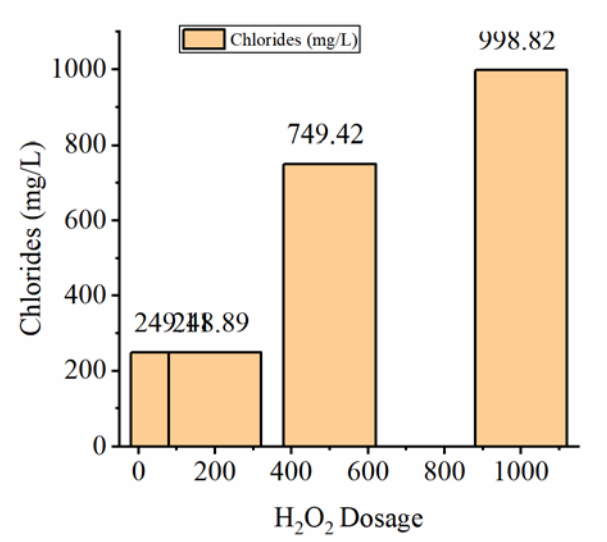


Figure 4. 39. Variation of chloride at 0.5 g/L of Jarosite loading and different H_2O_2 dosage Dicamba degradation.

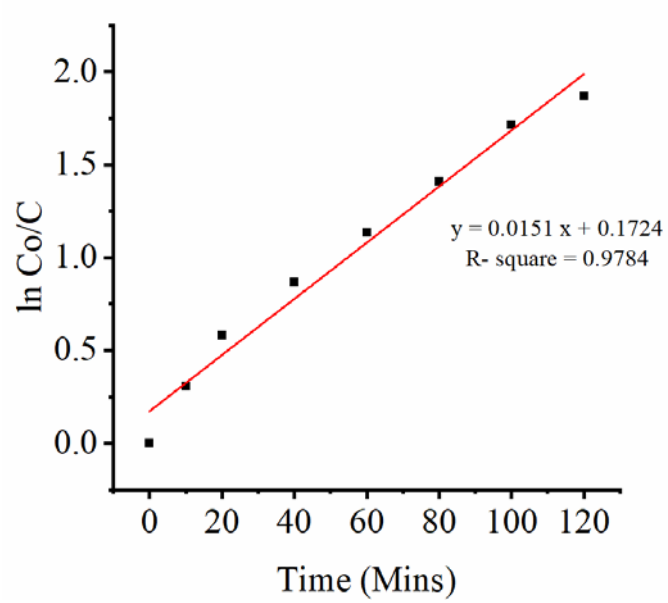


Figure 4. 40. Psuedo First order kinetic model fit ln C_o/C versus time

4.4.2 Catalytic Role of Bioleached Lateritic Iron (BLFe) in Fenton's degradation Selective Herbicides

4.4.2.1 Fenton's Degradation of Ametryn and Dicamba

Degradation of both ametryn and dicamba with bioleached lateritic iron catalyst by Fenton's oxidation process was evaluated separately with initial herbicide concentration of 5 mg/L and 100 mg/L respectively. The degradation slowly starts on the addition of H_2O_2 initiating the reaction. High removal efficiency up to 94.24 % was found with laterite iron dosage of 5 mg/L and H_2O_2 dosage of 50 mg/L for ametryn and 92.45 % with laterite iron dosage of 30 mg/L and H_2O_2 dosage of 300 mg/L for dicamba at pH 3.0 and temperature 30^o C. The COD removal was 88.04 % and 86.4 % in first 120 minute respectively. About 59.02 % removal for dicamba and 66.38 % for ametryn in the first 40 minute indicates better oxidation for both the herbicides. The experimental pH was kept constant varying the laterite iron dosage and H_2O_2 dosage. Increase in laterite iron dosage more than 30 mg/L and 5 mg/L for dicamba and ametryn reacts with the hydroxyl radicals hindering its role in the herbicide degradation phenomena. Similarly increase in H_2O_2 dosage from 100 mg/L to 200 mg/L, 300 mg/L and 400 mg/L for dicamba and 30 mg/L, 40 mg/L, 50 mg/L and 60 mg/L for ametryn did not shows any significant change with 300 mg/L and 50 mg/L bioleached laterite

iron load for dicamba and ametryn respectively. This may be due to no reduction in the induction period of process on H_2O_2 increase (Yan et al. 2017). Many research teams made successful attempt to extract iron from laterite soil chemically by acid digestion method and studied the catalytic role of extracted laterite iron in Fenton's process (Amritha and Manu 2016; Huang et al. 1993; Kumar and Gandhi 1990; Manu, B and Amritha A. 2018). Figure 4.41 demonstrates the degradation of ametryn via Fenton's oxidation. Bioleached laterite dose of 5 mg/L and corresponding H_2O_2 dosage of 50 mg/L was found to be effective catalytic load and hydrogen peroxide dosage with maximum degradation efficiency of 94.24 % at pH 3 and temperature 30 ^oC. However COD removal rate was 88.04 % in the first 120 minutes. During the treatment 66.38 % of COD was reduced in the initial 40 minutes indicating better oxidation rate. Increase in the bioleached laterite iron dosage more than 5 mg/L does not shows any significant increase in the degradation. This may be due to more iron reacts with the hydroxyl radicals hindering its catalytic in the herbicide degradation phenomena. Similarly increase in H_2O_2 dosage more than 50 mg/L does not contribute the degradation efficiency. Yan et al., (2017) conclude that in the Fenton's oxidation more H_2O_2 than need does not contribute to the reduction in the induction period of process on H_2O_2 increase (Yan et al. 2017). Figure 4.42 demonstrates the degradation of dicamba. Bioleached laterite dose of 30 mg/L and corresponding H_2O_2 dosage of 300 mg/L was found to be effective catalytic load and hydrogen peroxide dosage with maximum degradation efficiency of 92.45 % at pH 3 and temperature 30 ^oC. However COD removal rate was 86.4 % in the first 120 minutes. During the treatment 59.02 % of COD was reduced in the initial 40 minutes indicating better oxidation rate for dicamba degradation. Sangami and Manu (2018) studied the catalytic role of laterite extract iron nano particles in the degradation of herbicide dicamba and claim 100 % degradation (Sangami and Manu 2017; 2018). This supports the efficient application of laterite extract iron as a catalyst in the Fenton's oxidation process for the degradation of herbicides.

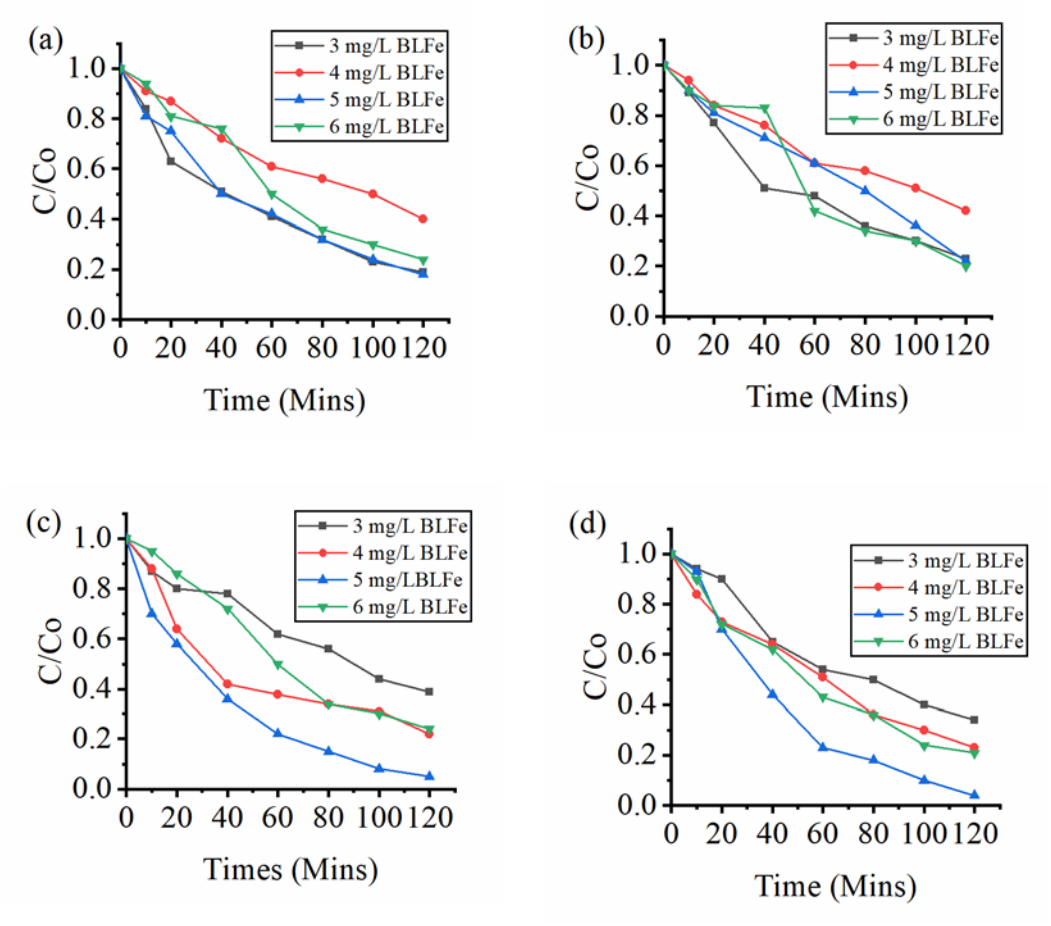


Figure 4. 41. Effect of Bioleached Laterite Iron (BLFe) loading on the process at different H_2O_2 concentration a) 30 mg/L H_2O_2 b) 40 mg/L H_2O_2 c) 50 mg/L H_2O_2 and d) 60 mg/L H_2O_2 for Ametryn degradation.

However the present study extends the scope of the previous work by making use of naturally available bacteria to extract the iron from laterite soil at zero cost. Basic pH condition does not provide workability in the Fenton's oxidation process. As the pH increases, hydrolysis results in the formation of ferric hydroxyl complexes which may cause the drop in degradation efficiency (Burbano et al. 2005; Kang, Y. and Hwang 2000; Khan et al. 2009). In the present study the no much variation in the pH was observed. The susceptibility of ametryn for Fenton's oxidation was observed even with the previous work. It is the presence of methylthio group in the ametryn makes it more amenable for hydroxyl ion attack (Bhaskar et al. 2019). Figure 4.43 demonstrates the COD removal during the process. The degradation follows pseudo first order reaction (Figure 4.44).

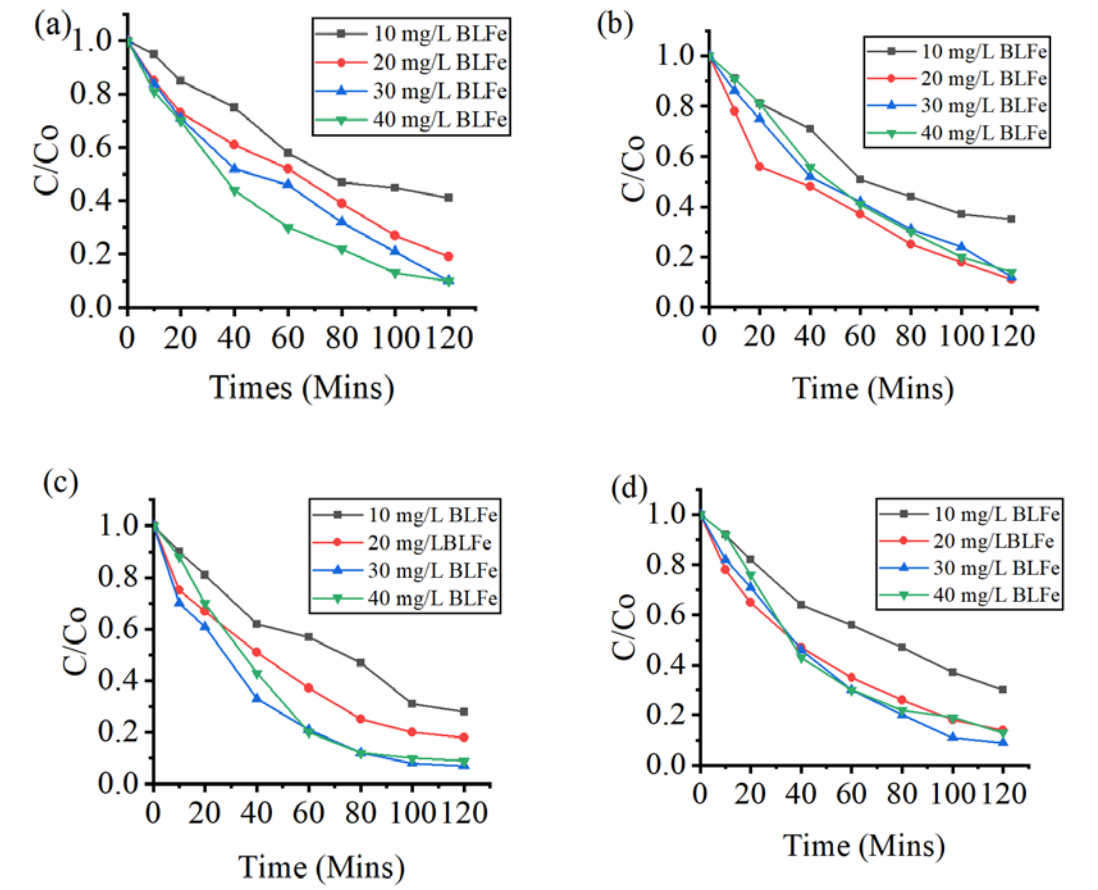


Figure 4. 42. Effect of Bioleached Lateritic Iron loading on the process at different H_2O_2 concentration a) 100 mg/L H_2O_2 b) 200 mg/L H_2O_2 c) 300 mg/L H_2O_2 and d) 400 mg/L H_2O_2 for Dicamba degradation.

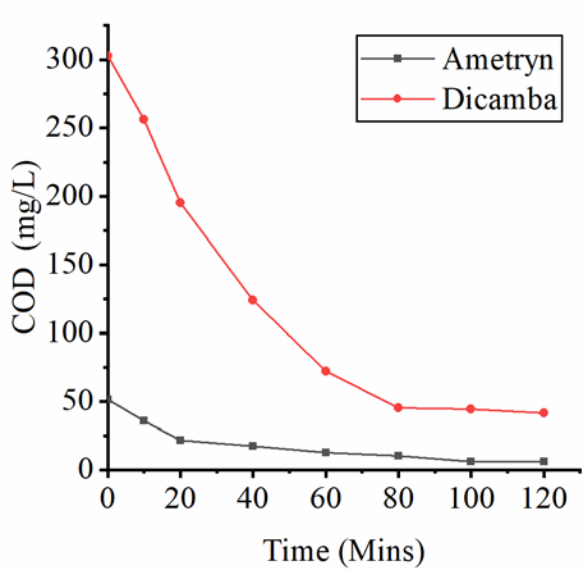


Figure 4. 43. Variation of COD during Fenton's Oxidation of Ametryn and Dicamba.

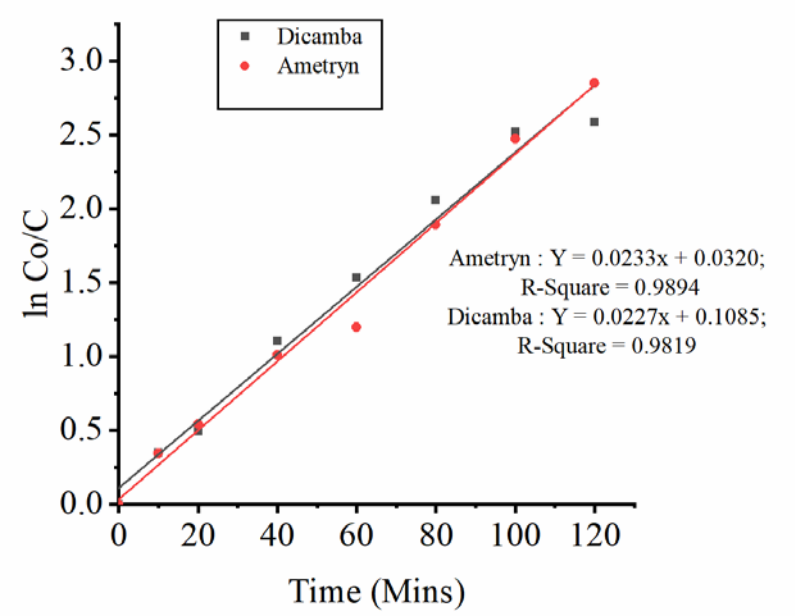


Figure 4. 44. Psuedo-first order kinetic model fit ln C/C_o versus time

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CHAPTER 5

CONCLUSIONS

1. An acidophilic novel bacterial strain was isolated and confirmed to be *Acidithiobacillus ferrooxidans*, a proteobacteria by the molecular techniques. Gene sequence of an isolated bacterial strain was submitted to Genbank and assigned an accession number **MG271840** by NCBI Genbank.

2. Phylogenetic analysis performed revealed that the isolated colonies are the members of acidophilic bacteria with autotrophic mode of metabolism. Isolated strain shows 94 % nearest match to ATCC strain ATCC23270 and 93 % match to ATCC strain ATCC33020.

3. Jarosite, an iron hydoxysulphate mineral was biologically synthesized in the laboratory using an isolated bacterial strain and characterized by X-ray diffraction and SEM analysis.

4. Catalytic efficiency of biologically synthesized jarosite was evaluated for its catalytic role in the Fenton's oxidation of selective herbicides ametryn and dicamba. Biojarosite catalyzed Fenton's degradation of ametryn shows upto 84.9% removal efficiency with the COD removal rate of 56.1% at 0.5 g/L jarosite loading and 100 mg/L of H_2O_2 dosage in first 2 h of treatment.

5. Biojarosite catalyzed Fenton's degradation of dicamba shows upto 91.29 % removal efficiency with the COD removal rate of 66.38 % at 0.5 g/L jarosite loading and 1000 mg/L of H_2O_2 dosage in first 2 h of treatment.

6. Bioleaching potential of an isolated strain *Acidithiobacillus ferrooxidans* BMSNITK17 (MG271840) was evaluated and confirmed for its active involvement in leaching process.

7. On shake flask studies various operating parameters were studied in regard to optimize the bioleaching conditions. Shake flask speed of 180 rpm was found to be optimum since this holds the bacteria in a solution in suspension providing a better contact of bacteria with the mineral ore.

8. pH in the range of 2.5 – 3.0 and temperature of 25 ^{o}C - 30 ^{o}C was found to be optimum for bioleaching of iron because of bacterial adaptation at this pH and temperature. Pulp density of 5% was found to be optimum in iron dissolution since this provides the better gas transfer thereby allowing the microbial metabolism to occur and particle size in the range 150 – 75 µm found to be optimum since higher particle size does not provides the bacterial attachment to mineral surface and smaller size gives a way to ferric precipitation coves the ore surface hindering the bacterial attachment. The drop in leaching efficiency after few days of inoculation was attributed to the formation of ferric precipitates and the high content of ferric in the study soil.

9. On sulphate supplement there was no significant improvement in bacterial iron leaching. It is the presence of gangue material and high ferric load on bacterial cells which inhibits the bacterial activity thereby halting leaching process.

10. Catalytic efficiency of bioleached lateritic iron was evaluated in the Fenton's oxidation of selective herbicides ametryn and dicamba. Overall degradation efficiency was found to be 94.24 % for ametryn and 92.45% for dicamba within two hours of the treatment respectively. The reaction follows pseudo first order reaction.

SCOPE FOR THE FURTHER WORK

On considering the study on bioleaching of iron from the laterite soil using novel isolated strain *Acidithiobacillus ferrooxidans*, the research extends its scope to study the adaptability of *Acidithiobacillus ferrooxidans BMSNITK17* extensively with respect to operating parameters in irol leaching from lateritic soil. The rate of iron dissolution is dropping after few days of inoculation providing high leaching rate the reason for this is attributed is ferric precipitation and high ferric load in the study soil which may be toxic to the bacteria. However, the claim needs in detail investigation which extends the further scope of the study. The present study is a laboratory scale shakes flask study which further gives a way for pilot plant lab scale studies for its industrial application in leaching iron and also insitu field studies with respect to operating parameters. The present study deals with the evaluation of bioleaching potential of one species of iron oxidizing bacteria *Acidithiobacillus ferrooxidans* and this extends its scope for further studies to deal with the other acidophilic iron oxidizing bacteria like *Leptospirillum ferrooxidans, Ferroplasms* etc.

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ANNEXURE I

REAGENTS FOR DNA ISOLATION

1. ***Lysis buffer preparation*****:** 2M Tris-HCl was prepared by dissolving 15.76 g separately in required volume of distilled water, pH was adjusted to 8.0 using 0.1N NaOH and the final volume was made up to 100 mL using distilled water. 0.1M EDTA was prepared by dissolving 37.22 g separately in requisite quantity of distilled water, pH was adjusted to 8.0 using 0.1N NaOH and the final volume was made up to 100 mL using distilled water. One hundred mL of 20% SDS was prepared using distilled water. 0.5M Lithium chloride was prepared by dissolving 0.105 g in 5 mL distilled water. The requisite quantities of 20% SDS, LiCl and PVP (as mentioned in Table) were dissolved and made up to 100 mL using distilled water. To this, 13 mL of 2M Tris-HCl, 34 mL of 0.1M EDTA were added and final volume of buffer was made up to 200 mL using distilled water.

Composition	Quantity
2M Tris-HCl	13.0 mL
0.1M EDTA	34.0 mL
20% SDS	83.0 mL
PVP	10.0 g
LiCl	20 µL
Distilled water (total volume of buffer)	200.0mL

2. ***50X TAE buffer preparation*****:** 242 g of Tris base was dissolved separately in 200 mL of distilled water. 0.5m EDTA.2Na was prepared by dissolving 18.5 g separately in required quantity of distilled water, pH was adjusted to 8.0 using 0.1N NaOH and the final volume was made up to 100 mL using distilled water. A known volume of glacial acetic acid was dissolved in required volume of distilled, pH 8.0 was adjusted using 0.1N NaOH and final volume was made up to 100 mL using distilled water. All the above solutions were mixed together and the final volume was made up to one litre using distilled water. A 2% of 50X TAE buffer was used as 1X TAE buffer.

Composition	Quantity
Tris base	242.0 g
0.5M EDTA.2Na	100.0 mL
Glacial acetic acid	57.1 mL
pH	8.0

***DNA loading dye (6X)*:** The requisite volumes of all the ingredients were dissolved in 50 mL of freshly prepared 1X TAE buffer and then the volume was made up to 100 mL and stored at 4 °C until use.

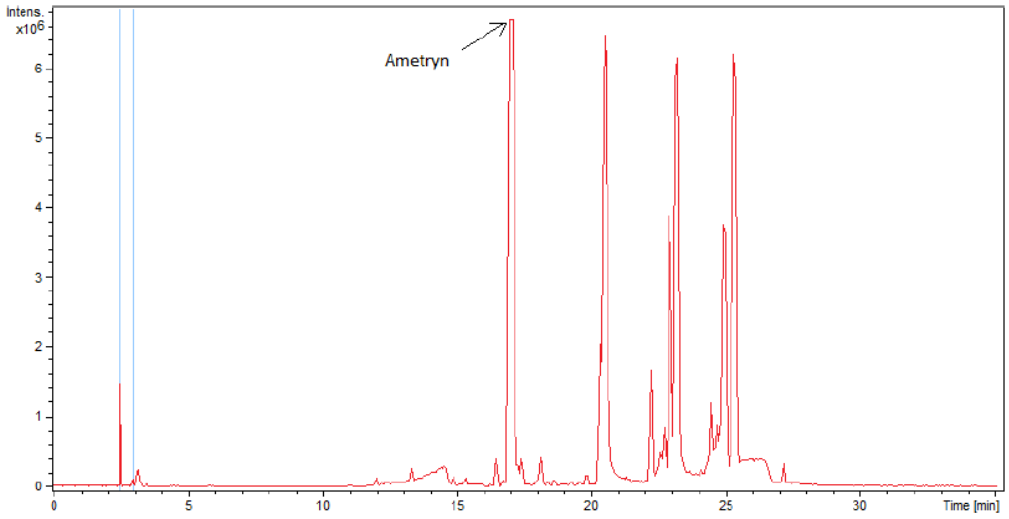
Composition	Quantity
Xylene Cyanol	0.25%
Bromophenol blue	0.25%
Sucrose/Gylcerol	40.0%

***Ethidium bromide solution*:** Stock solution of ethidium bromide involved 10 mg dissolved in 10 mL of distilled water. A working solution of 0.5 µg/mL was prepared and used in agarose gel analysis.

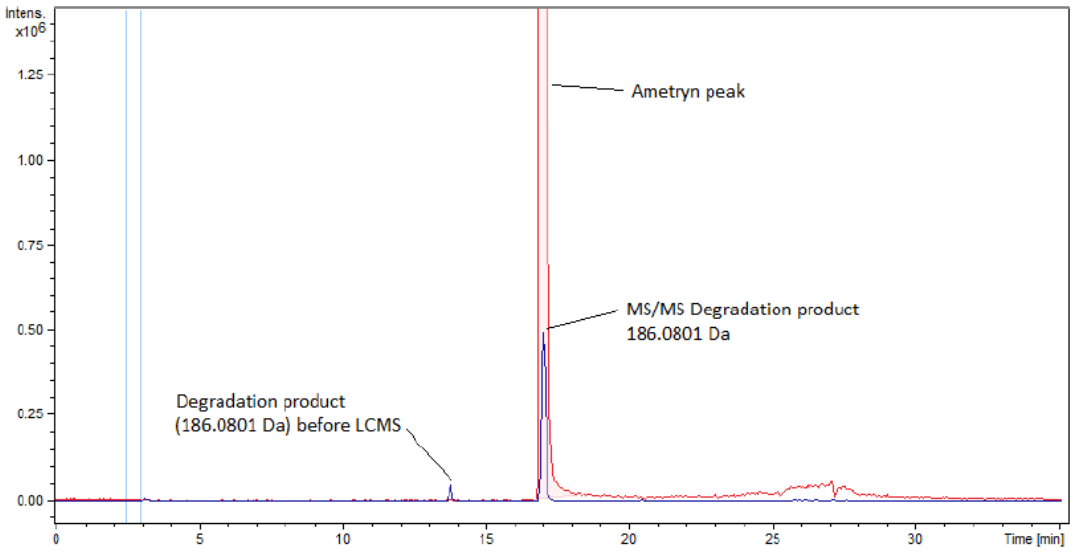
***Agarose gel preparation*:** 1.5% agarose gel was prepared by dissolving 0.75 g of agarose in 50 mL freshly prepared 1X TAE buffer, homogenized in a micro oven to completely dissolve agarose and allowed to cool to 50 °C. Then, 10 µL of ethidium bromide (working solution) was added, mixed gently to avoid air bubbles and poured into submarine electrophoretic chamber for further analysis.

ANNEXURE II

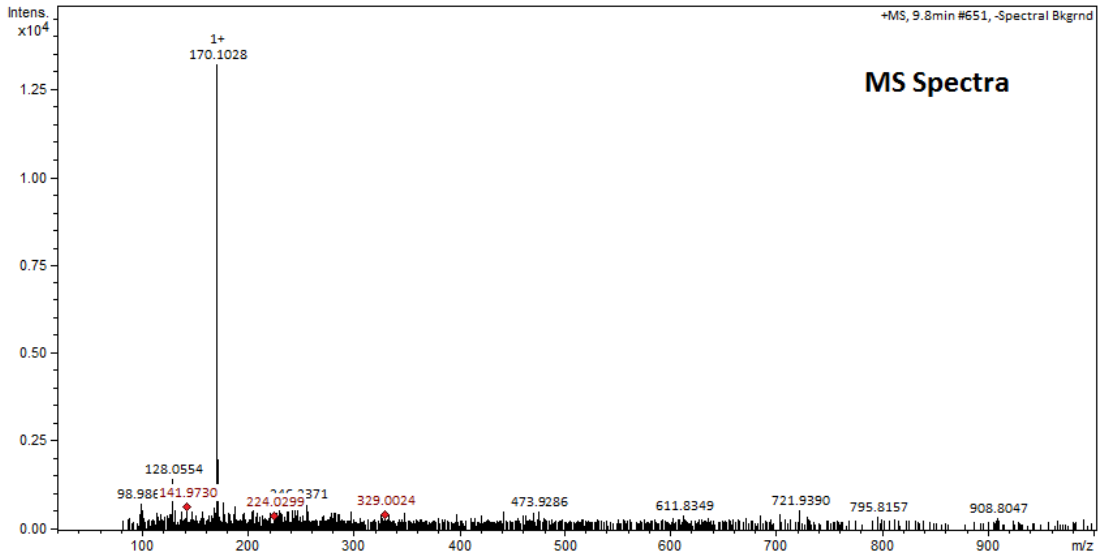
LC – MS CHROMATOGRAMS

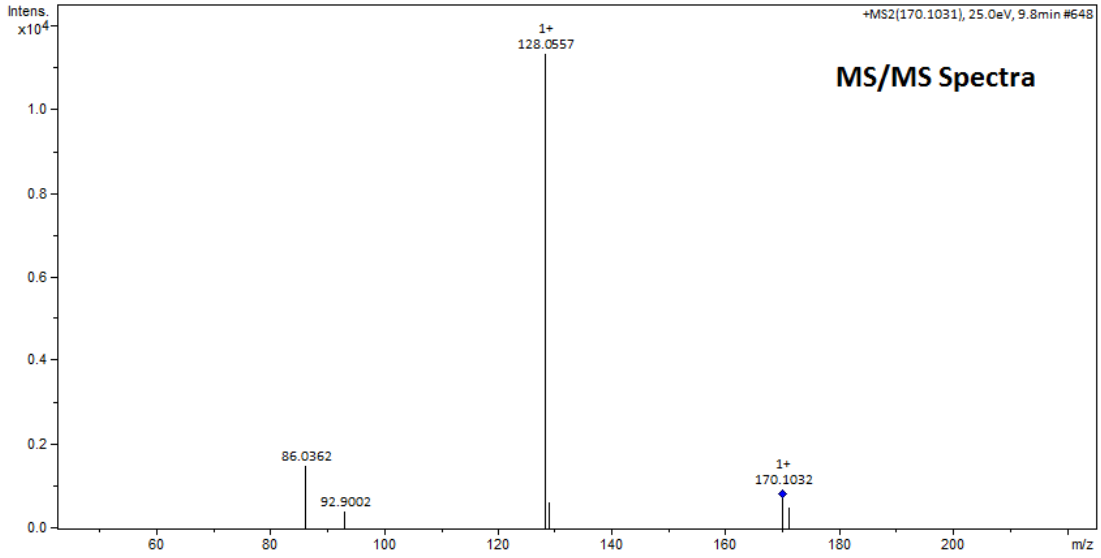


Basic Peak Chromatogram of LC-MS with standard Ametryn

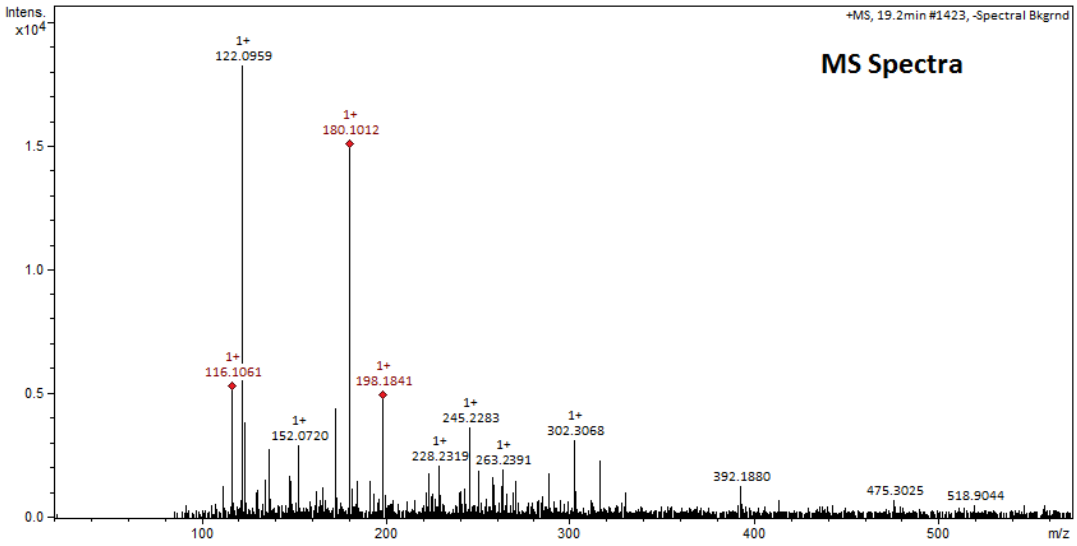


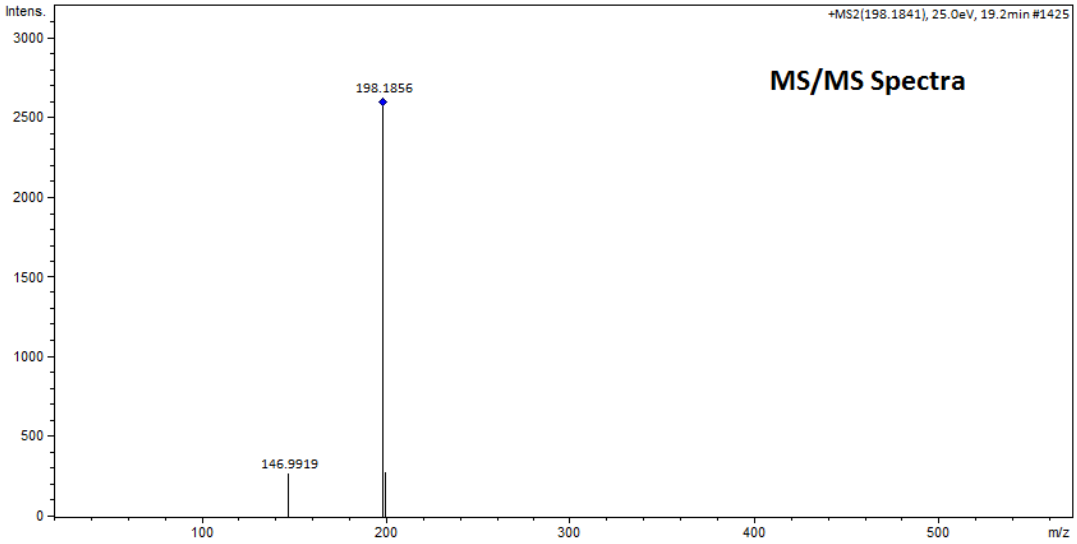
DEGRADATION PRODUCT 186.08 FROM AMETRYN





MS – MS/MS SPECTRA OF DEGRADATION PRODUCTS





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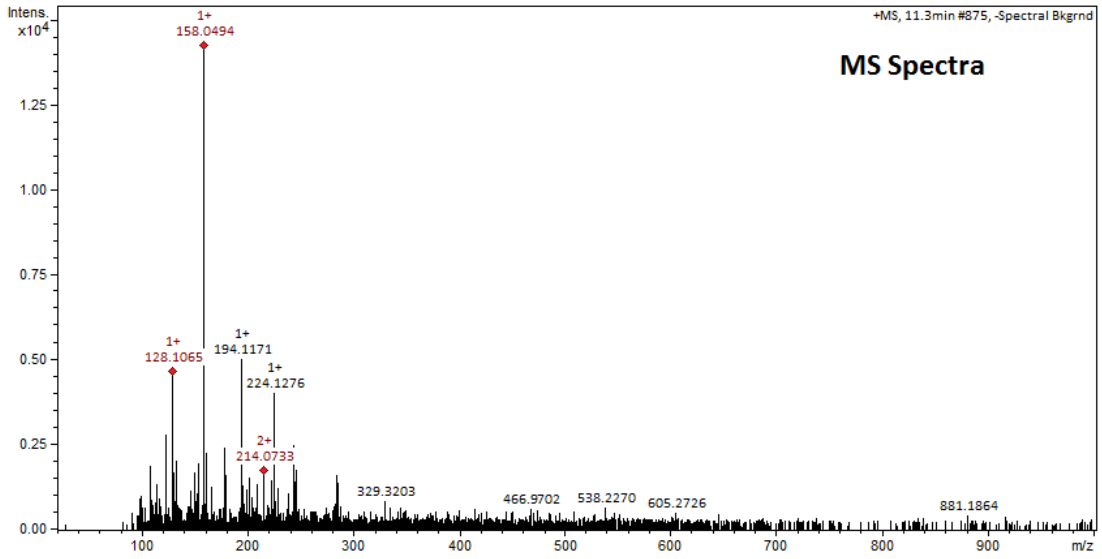

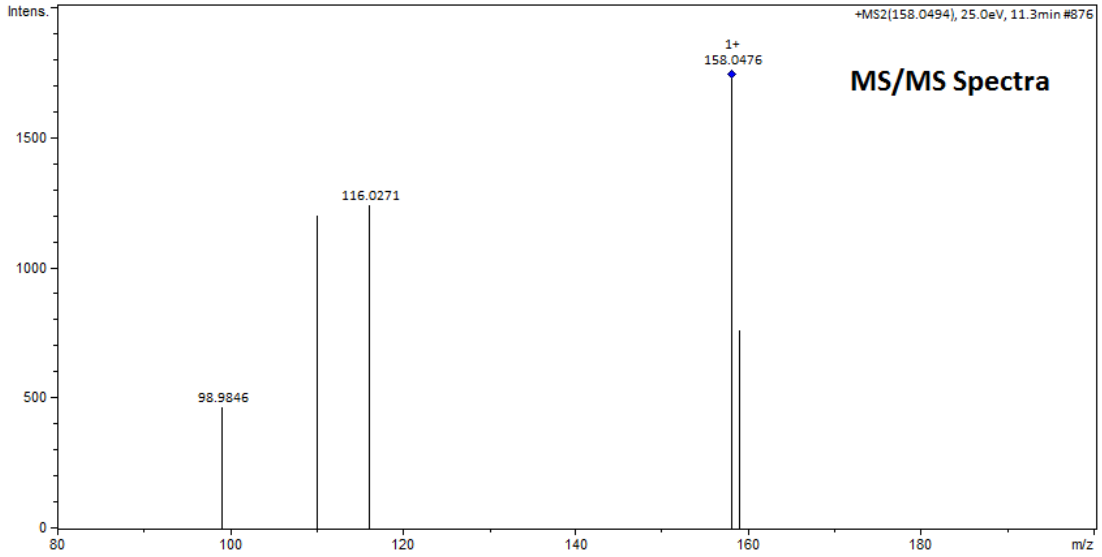

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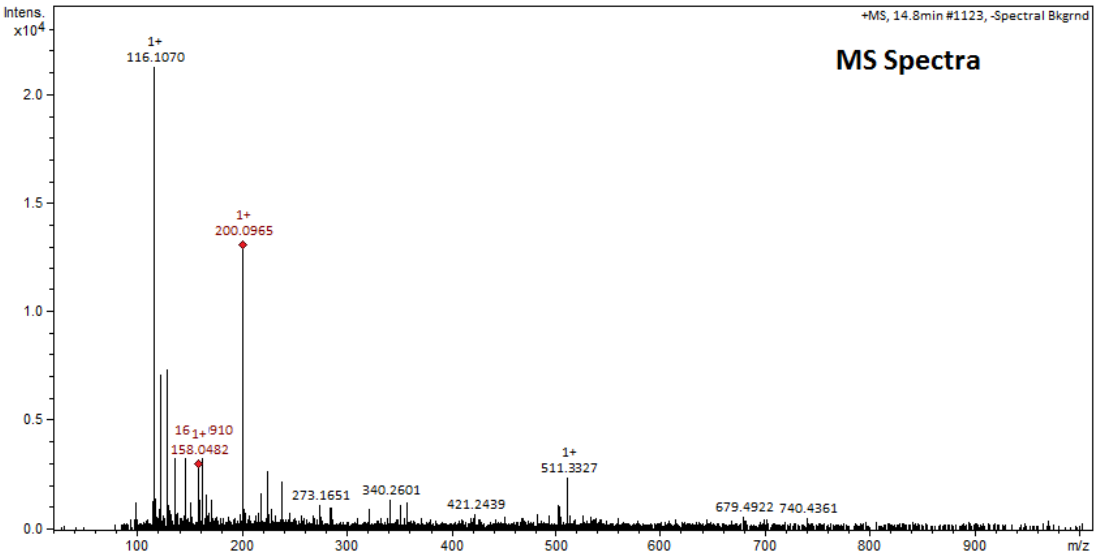

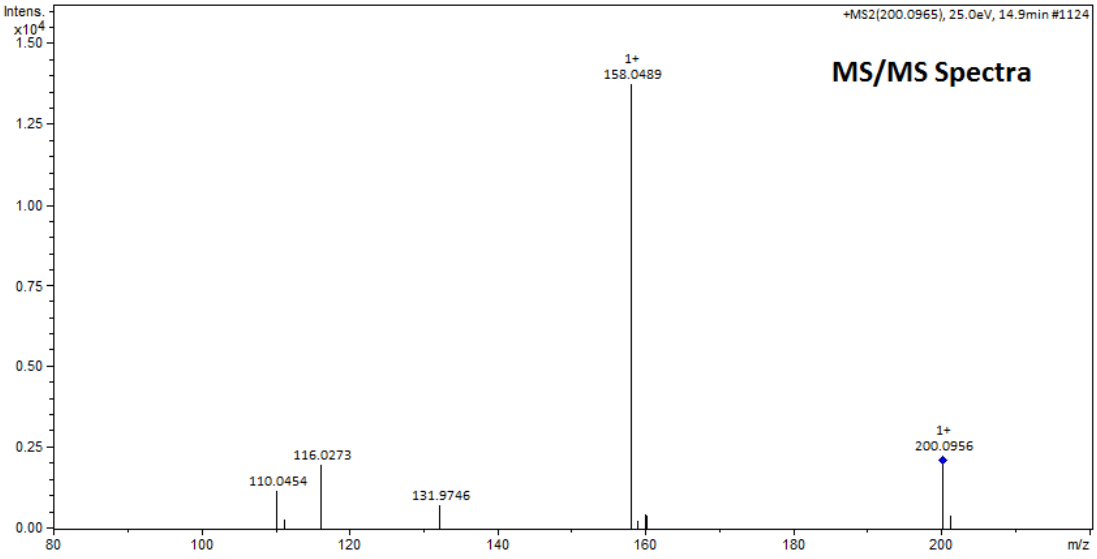

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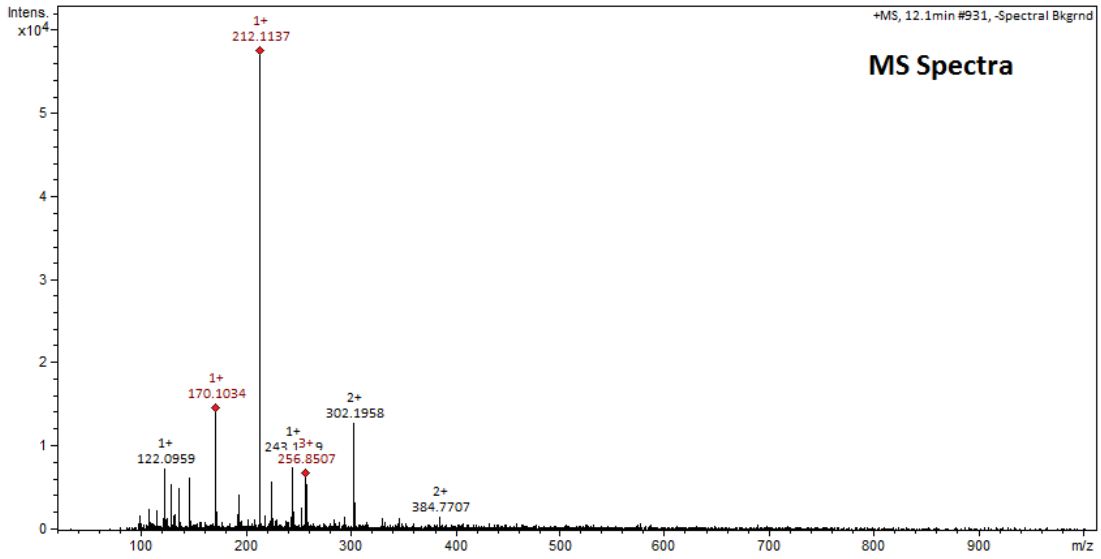
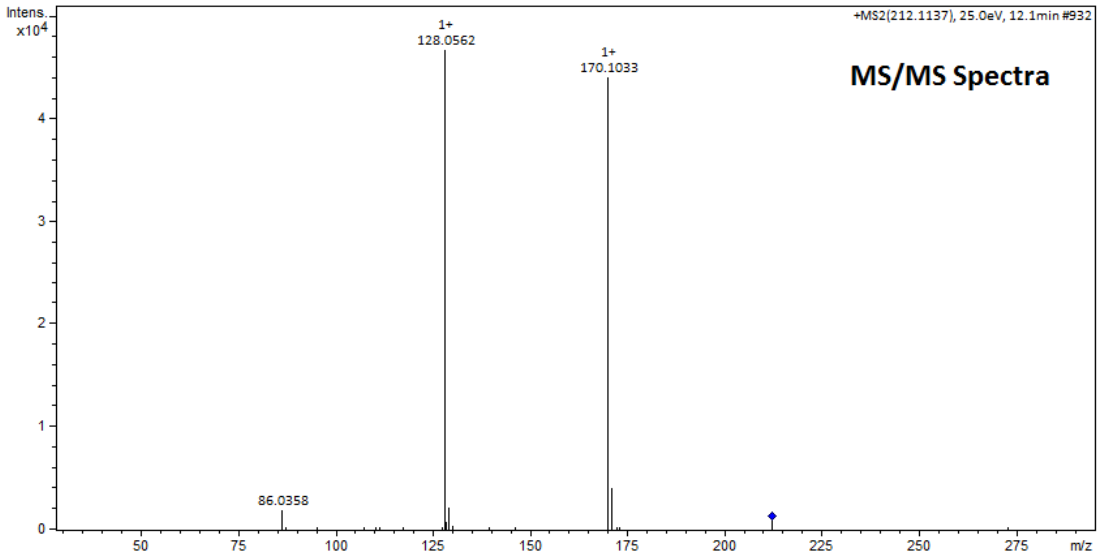




MS – MS/MS SPECTRA OF DEGRADATION PRODUCTS

ANNEXURE III

PLATES

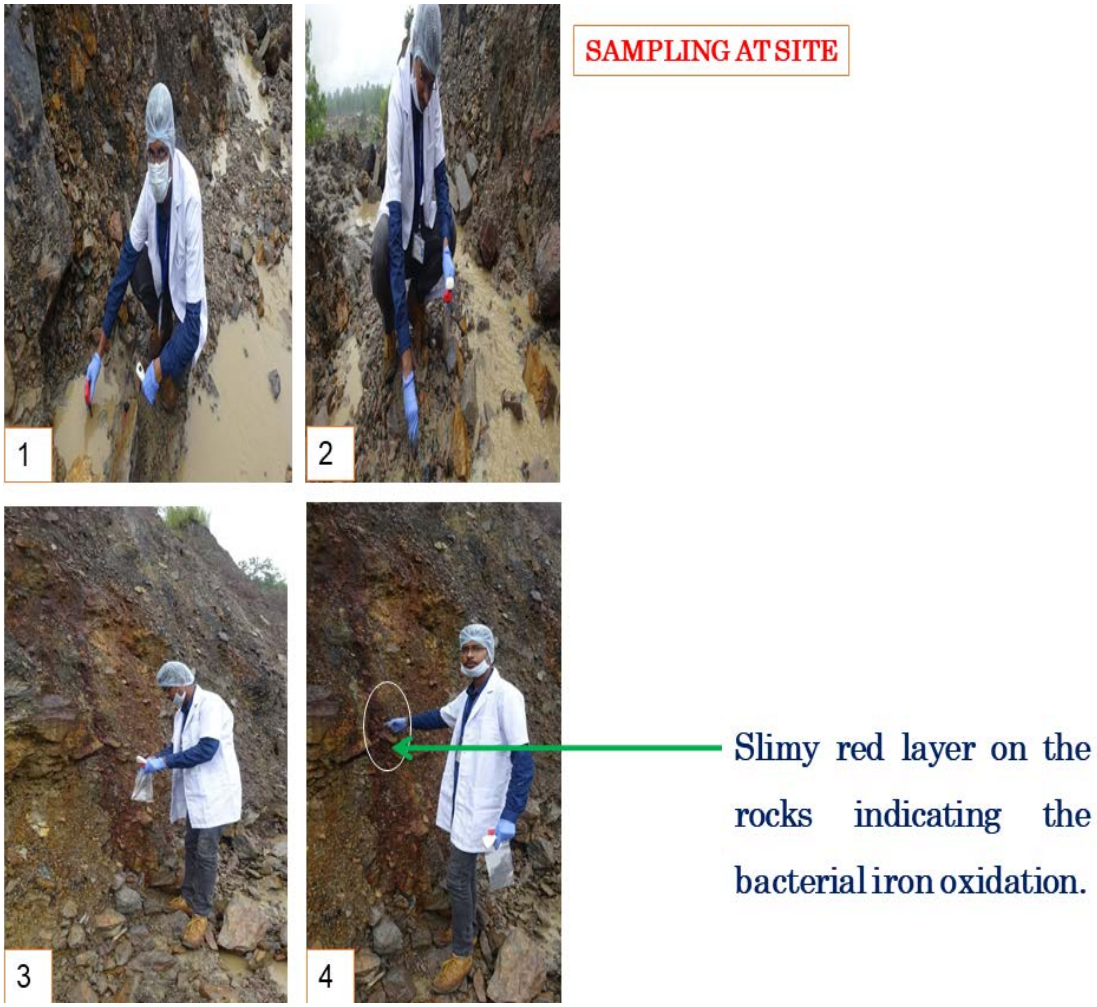


PLATE 1: SAMPLING AT AMD SITE

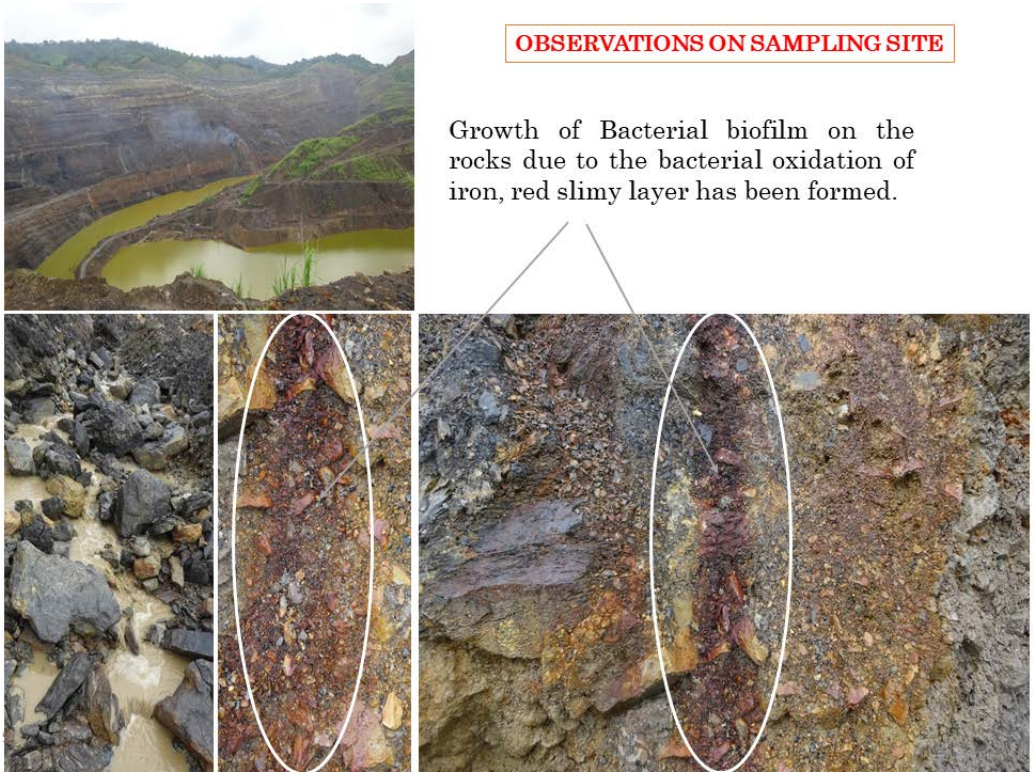
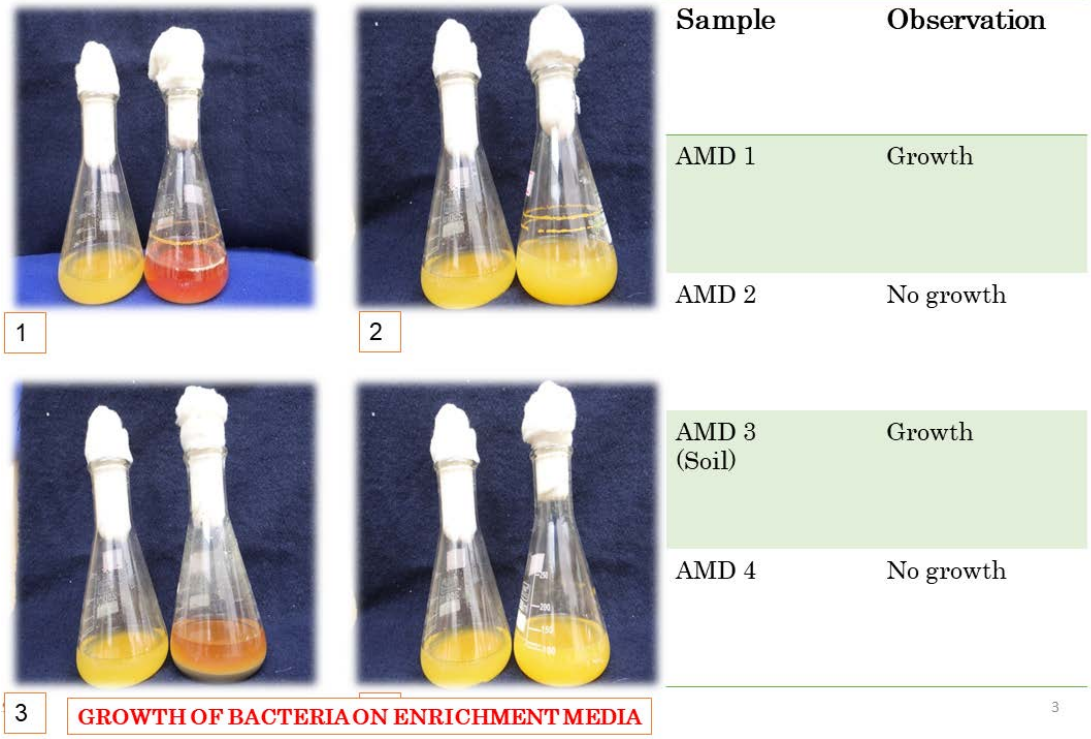


PLATE 2: OBSERVATIONS AT STUDY SITE



Sample	Observation
AMD 1	Growth
AMD 2	No growth
AMD 3 (Soil)	Growth
AMD 4	No growth

PLATE 3: BACTERIAL GROWTH ON ENRICHMENT MEDIA

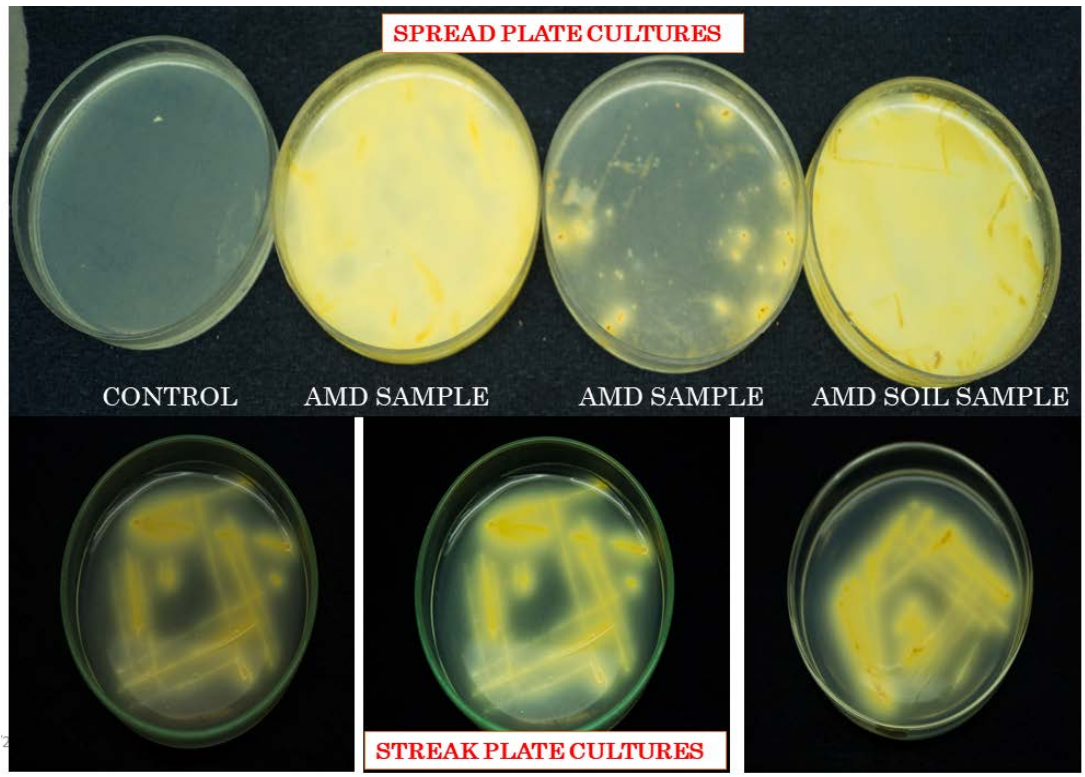


PLATE 4: BACTERIAL GROWTH ON SOLID MEDIUM

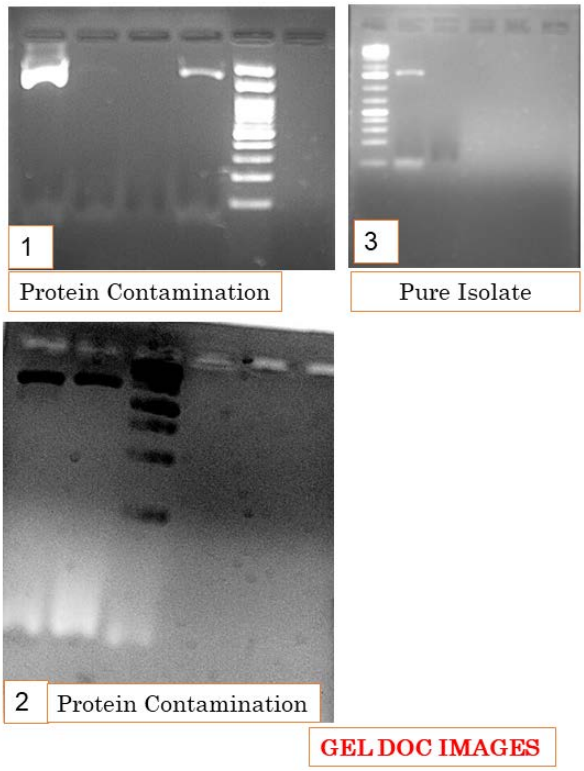


PLATE 4: GEL DOC IMAGE OF DNA OF ISOLATE

PLATE 5: EXTRACTION OF BIOGENIC JAROSITE

PUBLICATIONS BASED ON PRESENT WORK

International Journals Published

1. Bhaskar, S., Manu, B., & Sreenivasa, M. Y. (2019). Bacteriological synthesis of iron hydroxysulfate using an isolated *Acidithiobacillus ferrooxidans* strain and its application in ametryn degradation by Fenton's oxidation process. *Journal of environmental management*, *232*, 236-242.
2. Bhaskar, S., Manu, B., & Sreenivasa, M. Y. (2019). Evaluation of Catalytic efficiency of extracted iron from biosynthesized jarosite in the Fenton's oxidation of an herbicide Dicamba. *International Journal of Science and Innovative Engineering and Technology* (IJSIET).
3. Bhaskar, S., Manu, B., and Sreenivasa, M. Y (2020) "Bioleaching of Iron from fly ash using a novel isolated *Acidithiobacillus ferrooxidans* strain and evaluation of catalytic role of leached iron in the Fenton's oxidation of cephelaxin" *Journal of Indian Chemical Society (Accepted)*.

International Conference

1. Evaluation of catalytic efficiency of extracted iron from biosynthesized jarosite in the Fenton's oxidation of an herbicide Dicamba. *International Conference on Recent Engineering and Technology, New Horizon College of Engineering, Bangalore.*
2. Bhaskar S, Manu B & Sreenivasa M.Y (2019) "Green synthesis of Bioleached Laterite Iron Nanoparticles (GBLFeNP) using *Azadirachta indica* leaves and evaluation of its catalytic role in Fenton's oxidation of dicamba." *Conference on Nano-Micromaterials for circular economy and sustainability in the East Asia Pacific, Singapore.*
3. Bhaskar S, Manu B & Sreenivasa M.Y (2019) "Green synthesis of Bioleached Flyash Iron Nanoparticles (GFFeNP) using *Azadirachta indica* leaves and evaluation of its catalytic role in Fenton's oxidation of dicamba." International *Conference on Trending Moments and Steer Forces (TMSF-2019), Don Bosco Engineering college, Goa.*

4. Bhaskar, S., Manu, B., and Sreenivasa, M. Y (2020) “Bioleaching of Iron from fly ash using a novel isolated *Acidithiobacillus ferrooxidans* strain and evaluation of catalytic role of leached iron in the Fenton’s oxidation of cephelaxin” *International Conference CHEMBIOEN-2020, Dr. Ambedkar National Institute of Technology, Jalandar, India*.

International Journals- Under review-

1. Bhaskar, S., Manu, B., & Sreenivasa, M. Y. (2019). Bioleaching of iron from laterite soil using an isolated *Acidithiobacillus ferrooxidans* strain and application of leached laterite iron as Fenton’s catalyst in selective herbicide degradation. *Journal of Environmental Management.* (Under Review).
2. Bhaskar S, Basavaraju Manu, Nagaraja H, Deepa N, Deepthi B V, Rakesh S, M Y Sreenivasa (2019) Oxidative degradation of Dicamba using biosynthetic mineral catalyst Jarosite by Fenton’s process. *Bulletin of Environmental Contamination and Toxicology* (Communicated).