BIOMINING AND MICROBES

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Foreword

Mining is important for human being as it gives valuable minerals from the earth's crust for our betterment. Depending on the extraction processes the prices of minerals are decided in the international market. The novel discovery in the extraction process for minerals therefore plays an important role in the economy of the nation. The biomining is possible by using microbes from the soil with slow but constant rate and hence it is complimentary to the traditional methods of extraction. Biomining is sustainable too as it involves meagure amount of energy. The book is describing systematic and sequential methodology for the extraction of Iron and Aluminium metals. The improvement in the bioextraction is also suggested by generating mutants of microbes. I congratulate and wish all the best to authors.

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Preface

This book is the compilation of the Ph.D. thesis of Dr. Babasaheb Ambedkar Marathwada University, Aurangabad by Dr. Shaikh Shafikh Maheboob which was guided by Prof. Avinash B. Ade. We have tried to isolate the Thiobacillus ferroxidans from the bauxite ore from the Roha mines. Another bacterium was isolated from the ore was Pseudomonas fluorescesn. These are is the wonderful bacteria used for the biomining of the metals. After proper identification the treatment of these bacteria was given to the bauxite and coal fly ash. The performance was evaluated with various parameters under different environmental conditions. The strain improvement was also tried with the induced mutagenesis which was successful.

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Dr. Avinash B. Ade

Dr. Shaikh Shafikh Mahebub

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INTRODUCTION

The term, metal is applied to the substance that has a silvery luster and is a good conductor of electricity as well as heat. Some metals, like alkali metals are relatively soft, malleable and ductile. Alkaline earth metals and metalloids are the other two categories proposed by chemists. There are various methods of metal extraction from their mineral ore. Bioextraction of metal is an important process as it minimized the production cost of the metals.

Most of the microorganisms interact with metals. This property of microbes can be explored for metal extraction. The two important aspects can be discussed. These are concerning with the economic and industrial interest. First includes leaching of the metals from their ores and second involves leaching from the industrial wastes. In the first case, the metal is extracted from large quantities of low grade ores and in second reaction; metals are recycled and concentrated together. The later provides the tool for the removal of toxic and heavy metals; hence it helps in bioremediation of the polluted environment.

For many years the only microorganism thought to be important in the leaching of metals from the ores was the rod shaped bacterium, *Thiobacillus ferrooxidans*. It is strictly aerobic, Gram negative, polarly flagellated, motile and non-sporulating. It is able to oxidize sulphides, elemental sulphur, thiosulphate and polythionate. It is an obligate autotroph i.e. can not grow with organic carbon at neutral pH. It is responsible for acid mine drainage due to the metabolisms of coal piles causing the production of sulfuric acid. This bacterium was discovered in 1957 and onwards the correlation was established between the presence of this bacterium and the dissolution of metals in copper leaching operations.

Metal leaching begins with the circulation of water through large quantities of ores. The bacteria, which are naturally associated with the rocks, then cause the metals to be leached by one of the methods.

i) Either the bacteria act directly on the ore to extract the metal or ii) the bacteria produce substances such as ferric iron and sulfuric acid which later on extract the metal.

Thiobacillus ferrooxidans gets energy for growth from the oxidation of either iron or sulphur. The iron must be in the ferrous or bivalent form (Fe^{++}) and it is converted into trivalent (Fe^{+++}) ferric form by the bacterium. This bacterium also attacks several forms of sulphur, including soluble and insoluble sulphides, elemental sulphur and soluble compounds that incorporate either the thiosulphate or dithionate or the tetrathionate. In either case the product of the metal transformation is a substance in which the sulphur atom has fewer valence electrons, culminating in the formation of sulphate ions.

Other important leaching microbes are the moderately thermophilic and acidophilic bacteria grow on ferrous ions or minerals such as pyrites, chalcopyrite, covellite and Pentalandite. The bacterium Pseudomonas has several species associated with different functions in the degradation processes, along with these it can be used for the bioleaching of metals from mineral ores. In present investigation, metal accumulating and metal leaching property of the ***Pseudomonas fluorescens*** was verified.

The bacterial leaching is likely to find its greatest application in the controlled treatment of vast quantities of solid materials. From the solution, the microbes can remove metals in three steps, (i) absorption of metal ions into the surface of microorganism (ii) the intracellular uptake of metals and (iii) chemical transformation of metals by biological agents. Microbes posses a negative electric charge, because of this property most positively charged metal ions from the solution are adsorbed on the cell surface.

The direct leaching involves an enzymatic attack by the bacteria on components of the mineral that are susceptible to oxidation. In the process of obtaining energy from the inorganic material, the bacteria cause electrons to be transferred from iron or sulphur oxygen. In principle the inorganic ions do not enter inside the bacterial cells while electrons released due to oxidation process are transported through a protein system in the cell membrane and these form the oxygen atom forming water. The transferred electrons give up energy which is coupled to the formation of adenosine triphosphate. The extraction of metals is carried out in presence of water therefore; the whole branch to study this type of reactions is described as biohydrometallurgy. In the biohydrometallurgical process the role of Thiobacillus and other bacteria is dominant while in the bioleaching of metals from wastes, the fungal species Saccharomyces cerevisae, Rhizopus arrihizus and many species of Pseudomonas play a key role for accumulation of metals.

To implement the biomining processes, the environmental factors are also considered. Metal leaching microbes reproduce when they have abundant energy source and optimal conditions like sufficient oxygen, carbon dioxide and an acidic environment. The logarithmic multiplication of microbes occurs until lack of food or oxygen or an unfavorable pH prevents the organism from further cell division.

In extreme environment biomining can be effectively used. The topographical factors, such as high altitude do not affect the process; however, an additional air must be supplied to the organisms to provide them sufficient oxygen for optimal performance, because at high elevation, the partial pressure of oxygen in the air decreases. Cold temperature slows both the rate of microbial activity and the break down of the sulphide mineral; however, breakdown of sulphide minerals produces a lot of heat. Therefore, at high temperatures the biomining gives good results of metal extraction. But in extremely thermophilic condition the mesophilic (ambient temperature) microorganisms are killed.

The other affecting factors for bioleaching are mineral ore texture, moisture, pH and temperature etc. Associated with this, the byproducts of bioleaching organisms are considered. Very less attention has been given to other bioleaching microbes as compare to *Thiobacillus ferrooxidans*. Therefore there is a need to explore more genera for bioextraction of metals.

There are two main types of processes for commercial scale microbial assisted metal recovery. These are irrigation type and stirred tank type processes. Irrigation process involves the percolation of leaching solution through crushed ores or concentrates that have been stacked in columns, heaps or dumps while stirred tank type process employ continuously operating, highly aerated stirred tank reactors. One feature of both the types of processes is that, unlike most other commercial fermentation processes, neither is sterile and no attempt is made to maintain the sterilization of the inoculum. There is no need of sterilization because the highly acidophilic chemolithotrophic microorganisms create an environment that is not suitable for the growth of other organisms. Second, because the aim of the process is to maximize the decomposition of the mineral; the microorganisms that do this most efficiently are selected, as compare to those that are less efficient. There is a continuous selection for those organisms which are most effective in decomposing the mineral, as the mineral provides the only energy source available.

Bauxite deposits of India are of diverse percentage. It is the main source of aluminum and iron. Aluminum is the second most abundant metal element in the earth's crust after silica. The bauxite deposits are of laterite type. Bauxite is a naturally occurring heterogeneous and is composed chiefly of aluminum oxide minerals as gibbsite, the trihydrate bohemite, diaspore ($Al_2O_3.H_2O$) and monohydrates. The bulk of world bauxite production (approximately 85 %) is used as feed for the manufacture of alumina via a wet chemical caustic leach method commonly known as Bayer's process which has remain unchanged since its discovery in 1888.

Fly ash is the incombustible matter which is left after all the organic components of the coal have been consumed during the process of coal combustion and is collected by means of mechanical electrostatic precipitators. Fly ash is composed of mainly silt-sized spherical amorphous ferro-aluminosilicate minerals and is generally characterized as having low permeability, low bulk density as well as high specific surface area. The ash particle generally ranges in size from 0.5 to 200 µ. Fly ash particles are mainly composed of silicon (SiO_2), aluminum (Al_2O_3), iron (Fe_2O_3), titanium (TiO_2), and mangnese (Mn_2O_3) (Scheetz and Earle, 1998).

Aluminum is applicable in diversified areas. It is useful in industries like aviation, as it is lighter metal. Common house hold utensils can be prepared from aluminum. Aluminum foils are used for wrapping the food in the restaurents. The available processes for the extraction of the aluminum are based on providing electric current to reduce dissolved aluminum ions. It is expensive, therefore there is a need to develop the method which involve bioextraction and effective subsequent treatment as step wise process involving both of these or bioextraction alone.

In order to minimize the toxic effects of aluminum from the industrial wastes to the plant and animals, it can be diverted for precipitation and accumulated as aluminum metal.

The metal, iron is widely used in heavy infrastructure of industrial set up. For construction purpose its role is enormously important. It is equally important in agricultural engineering, in defence and in household appliances of daily use.

In view of this background and need, the present work of investigation was undertaken. The first part involves isolation of the effective isolates of bioleaching microbes from bauxite ores and industrial waste (fly ash) collected at dumping sites. The micro flora of these sources was tested. The mineral ore and flyash were analyzed by XRD and EDAX / EDX. In second phase the isolated bioleaching microbes were treated with bauxite ore and fly ash using various parameters. The estimation of aluminum and iron was done by spectrophotometry using appropriate dyes and the amount was compared.

The FTIR (Fourier Transform Infrared Spectroscopy) was done for the analysis of treated and non treated bauxite ore and flyash. In infrared spectroscopy, IR irradiation (10,000 - 200 cm^{-1}) is passed through a sample. A part of the radiation is absorbed by the sample or transmitted through it. An infrared spectrum represents a fingerprints of a sample with reference to the absorption peaks which corresponding the frequencies of vibrations between the bonds of the atoms making up the compound. The occurrence of a vibrational transition in the infrared region is dependent on an overall change of the electric dipole moment during the particular vibration.

In third step for increasing the efficiency of metal leaching, the genetic improvement of the isolates of metal leaching microbes was carried out by mutation and the better mutants were selected for further sequential steps in order to make the process well defined for industrial use. The analysis of mutants was carried out using RAPD technique with 8 primers. Out of 8 primers, 4 primers were found successful for the DNA amplification. The protein profile during metal extraction process was done by SDS-PAGE.

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REVIEW OF LITERATURE

Mineralization is the process in which the elemental carbon, nitrogen, sulphur, phosphorous and other trace elements are released in the environment by soil microorganisms from organic material. The microbes are considered as important geologic agents playing a role in processes such as mineral formation, mineral degradation, sedimentation, and weathering (geological cycling).

Recovery of metals has been quiet an ancient practices observed in the Mediterranean Basin as early as 1000 B.C. (Brierley, 1982) Even in Spain, in the 18th century, leaching of copper was being done at Rio Tinto mines. However the microbial interaction and involvement in leaching was realized 50 years ago.

BIOHYDROMETALLURGY

Biohydrometallurgy is the offspring of the unexpected union of biotechnology and metallurgy from specific properties of some extreme biotopes, active principles of interaction between microbial metabolism and minerals have been explored to be used as efficient metallurgical processes. Microbes encounter metals and metalloids of various kinds in the environment and these are therefore interacting with them, sometimes to their benefit, or at other times to their detriment.

The main application of biohydrometallurgy at industrial scale is bioleaching. It consists in dissolving metal sulphides by the use of bacterially assisted reactions. More preciously, it uses the catalytic effect produced by the metabolic activity of some iron-oxidizing and sulphur oxidizing micro-organisms results in an acceleration of the chemical degradation of the sulphides. There are two ways of applying bioleaching for the recovery of metals from sulphide ores and concentrates, namely tank leaching and heap leaching (Rawlings and Johnson**,** 2007).

Although such operations are difficult to document, it is known that the leaching of copper on a large scale was well established at the Rio Tinto mines. The microbes help to convert the copper into water soluble form that can be carried off by the leach water. Today bacteria are being deliberately exploited to recover millions of pounds of copper from billions of tons of low grade ore. In recent years bacterial leaching has been applied to the recovery of non-ferrous metal, such as uranium (Brierley, 1982).

Microbial leaching of metals from sulphide minerals has been practiced over hundreds of years without realization that microorganisms were involved (Torma, 1977). General conditions for bacterial leaching as well as analysis of process and mechanisms have been reviewed and discussed in books and various proceedings of the conferences (Bosecker, 1997, Rawlings and Silver, 1995, Torma, 1977). There are examples of commercially successful metal leaching including the recovery of copper, uranium and gold (Bosecker, 1997).

The way, microbes interact with metals, depends in part on whether the organisms are prokaryotic or eukaryotic. Both types of microbes have the ability to bind metal ions present in the external environment at the cell surface or to transport them into cell for various intracellular functions. On the other hand, only the prokaryotes include organisms that are able to oxidize or reduce on a large scale and conserve energy in their reactions. Some microbes may reduce metal ions such as Hg^{2+} and Ag^{+} to Hg and Ag respectively but do not conserve energy from these reactions. Some prokaryotes and eukaryotes may form metabolic products, such as acids or ligands, which dissolve base metals contained in minerals such as Fe, Cu, Zn, Ni, Co, and others. Others may form anions such as sulphides or carbonate, that precipitate dissolved metal ions. Some prokaryotes may methylate some metal and metalloid compounds, producing corresponding volatile metal derivatives (Ehrlich, 1997).

The two possible mechanisms whereby microorganisms can increase the leaching rate of metals from mineral ores are that due to purely physico-chemical processes. These are direct or indirect actions of microorganisms (Suzuki, 2001). In direct action, microorganisms oxidize minerals and solubilize metals. While In indirect action of microorganisms, ferric ion is the oxidizing agent for minerals and the role of microorganisms is simply regeneration of Fe^{3+} from Fe^{2+}**.** However, in the actual microbial leaching of metals, both direct and indirect leaching as well as physico-chemical reactions can contribute. Recently there has been a serious discussion on the involvement of direct action by microorganisms in mineral leaching. Ehrlich (1997) cited a number of references in support of the direct cell attachment on surface of minerals and the role of Fe^{3+} bound on the cell surface for sulphide oxidation. Sand et al. (1995) proposed that bacterial leaching of metal sulphides proceeds essentially by indirect mechanism initiated by ferric ion Fe^{3+}. In their definition of indirect action, however, they included the role of Fe^{3+} bound by extracellular polymeric substances (Gherke et al., 1998), which was essential for binding of cells to pyrite. They further proposed two indirect oxidation mechanisms for the sulphur portion of metal sulphides after the initial attack by Fe^{3+} (Schipper and Sand, 1999).

Devasia and Natrajan (2004) emphasized on the use of bacteria for leaching because worldwide reserves of high grade ores are diminishing at an alarming rate due to the rapid increase in the demand for metals. However there exist large stocks of low and lean grade ores yet to be mined. The problem is that the recovery of metals from low and lean grade ores using conventional techniques is very expensive due to high energy and capital inputs. Another major problem is environmental costs due to the high level of pollution from these technologies. Therefore they have concentrated on the two major techniques used in leaching which are percolation and agitation leaching. Percolation involves the percolation of lixiviant through a static bed, whereas agitation leaching involves finer particle sizes agitated in a lixiviant. Due to the large scale operation involved in bacterial leaching, percolation leaching is preferred commercially.

Brierley (1982) have categorized bioleaching process into four parts. Dump bioleaching, heap bioleaching, heap mineral biooxidation and stirred tank mineral biooxidation. Bioextraction of metal was worked out by several other authors like Heinen and Lauwers (2005) who have studied the extraction of silica and uranium from radioactive thermal spring. Mishra et al. (2004) have studied the bacterial leaching of metals from sulphide minerals and industrial wastes. From polish Black shale metals were also extracted (Farbiszewskia et al., 2004). The biofloatation of the low grade Sarchesmeh copper sulphide was studied by Kolahdoozan et al. (2004). Bioleaching of zinc and aluminum from industrial waste sludge was studied by Solisio et al. (2002). Silver, gold and lead recovery from bioleaching process especially from the bioleached residue was also analyzed by Frias et al. (2002). The production of alum was worked out from bauxite by Francis et al. (1999).

Almost all microorganisms interact with metals. Two important sections that are of potential economic and industrial interest are leaching metals from their ores and secondly they can be recycled from originally existing metal products or the pollutants containing metals.

An ore is a solid and naturally occurring mineral aggregate of economic interest from which one or more valuable constituents may be recovered by several treatments (Trehan, 1996).

BAUXITE

The description of bauxite components was provided by Lancashire (1982). According to classic Encyclopedia which is based on the 11th Edition of the Encyclopedia Britannica. This name being derived from the original locality, the Village of Les Baux (or Beaux) near Arles, dep. Bouches-du-Rhone in the South France, where the material has been for many years extensively mined as an ore of aluminum. It was found in earthy clay like masses. In colour it

varies from white through yellow and brown to red depending on the amount of and the degree of hydration of the iron present.

Bauxite is a naturally occurring heterogeneous ore, composed chiefly of aluminous oxide minerals as gibbsite ($Al_2O_3.3H_2O$), the trihydrate bohemite, diaspore ($Al_2O_3.H_2O$) and monohdydrate etc. Major impurities in the Bauxite ore are iron oxide, silica, aluminum silicates and titanium oxides. Free moisture in crude bauxite as mined may range from 5 to 30%. Other aluminum resources for future include Kaolinitic clays, Nepheline Syenite, Anorthosite and Alunite. Bauxites are typically classified according to their intended commercial application: abrasive, cement, chemical, metallurgical refractory etc. The bulk of world bauxite production (approximately 85 %) is used as feed for the manufacture of alumina via a wet chemical caustic leach method commonly known as the Bayer's process. (Choudhary, 2007). The bauxite ore is very hard and massive in the top and moderately hard and spongy below. The pale buff to creamy white colored bauxite is mostly of high grade and is recorded mostly on surface. Bauxite, pale pink in colour and with a changing appearance, has been found to occur towards the lower part of the bauxite zone. Crystalline crypto crystalline and gibbsite occur in varying proportion. The porosity and moisture contents are highly variable. Specific gravity ranges from 2.1 and 2.5 (Choudhary, 2007).

ALUMINUM

In the bauxite the aluminum is found in maximum quantity and it's the most abundant metal in the Earth's crust, but it is usually bound in insoluble oxides and silicates. Environmental acidification can increase levels of soluble aluminum [Al (III)] is known to be toxic to many organisms. No biological role has been identified for aluminum, but aluminum can replace hydrogen with archaea. Research into microbial interaction with aluminum has focused on corrosion of aluminum alloys, leaching of aluminum from materials and aluminum accumulation, toxicity and detoxification. Organic and inorganic acids produced by bacteria and fungi can mobilize aluminum from a variety of materials. Corrosion of aluminum alloys due to the growth of microorganisms was observed in aircraft fuel tanks in the 1960's and remains a current area of research. Researchers have documented microbially mediated aluminum leaching from the mineral spodumene (Karavaiko et al., 1980), red mud waste remaining after the alkaline extraction of alumina from bauxite ores (Vachon et al., 1994), and incinerator fly ash (Brombacher et al., 1998) from municipal waste incineration is a concentrate containing a wide variety of toxic heavy metals. The zinc concentration in the fly ash can be similar to the concentration in ores subjected to conventional mining. The coal fly ash was shown as aluminum and iron sources by Seidel et al. (2001).

MICROBES FOR BIOLEACHING OF ALUMINUM

Phosphate has been shown to influence the mechanisms of aluminum tolerance in ***Pseudomonas fluorescens*** grown with aluminum-complexed citrate as a sole carbon source. In a phosphate-rich medium ***Pseudomonas fluorescens*** deposited aluminum in an insoluble extracellular residue composed partly of phosphatidyl ethanolamine (Apparna and Pierre, 1996). Aluminum detoxification mechanism is used by **Pseudomonas fluorescens**. Cellular phosphate concentration can also modulate aluminum toxicity in Bradyrhizobium japonicum.

Many microbes are known for bioextraction or bioleaching of the metals. The sulphur consuming bacteria *Thiobacillus ferrooxidans* is one of them. The phenotypic characteristics of *Thiobacillus ferrooxidans* showed by studying its strain from different ecological niches. The bacterium has been placed in the order Acidithiobacillales and the genus is revised on *Acidithiobacillus* and placed in Acidithiobacillaceae.

The *Acidithiobacillus* was named by Kelly and Wood (2000). However, in routine work Thiobacillus is a common term. The genus has four species Thiobacillus thiooxidans which

grow as minute colonies on thiosulphate agar. Thiobacillus albertensis grow on sulphur, thiosulfate or tetrathionate etc. The species Thiobacillus caldus is an again a chemolithotroph grow on thiosulfate, tetrathionate, sulphur and molecular hydrogen while there is no growth on ferrous iron or sulphide ores. *Thiobacillus ferrooxidans* are rods, usually single or in pairs on 0.5 x 10 µm, motile, with a single flagellar, small colonies grown on thiosulfate or tetrathionate agar. The colonies are round sometimes with irregular margin and white with sulphur on solid media with ferrous sulphate. Liquid medium with ferrous sulphate at pH 1.6 changes from clear pale green to amber to red-brown with ferric sulphate. It is strictly aerobic; grow at 10-37 ^{0}C. The G+C content of the DNA is 58-59 (Tm). The metal tolerant strains of *Thiobacillus ferrooxidans* are ferrous tolerant. The original isolation and studies of the iron oxidizing bacteria presently assigned to the genera Thiobacillus and Ferrobacillus were carried out in acid media rich in sulphate ions (Colmer and Hinkle, 1947; Temple and Colmer, 1951). Although these appears to be some conflict as to whether all of these three autotrophic iron oxidizing bacteria can produce sulphate as the result of biological oxidation of sulphide, thiosulphate or sulphur, (Lazaroff, 1962). Extensive work has been done on *Thiobacillus ferrooxidans* in presence of sulphate it is autotrophic. The autotrophic nature of it could be elaborated by evidence for the Calvin cycle and hexose monophosphate pathway. The autotrophic growth of *Thiobacillus ferrooxidans* was demonstrated by Tuovinen and Nicholas (1977).

Along with this its heterotrophic transition was also studied. The heterotrophic nature was observed on glucose. The growth of *Thiobacillus ferrooxidans* on solid medium using purified agar, carrageen and agarose was studied. The strain produced isolated and transferable colonies after 7 days of incubation. Growth (increase in the number of viable cells) by direct plating method was followed in relation to iron oxidation. Acidity, agar concentration and phosphate influenced colony development on solid medium was also observed (Mishra and Roy, 1979). Growth kinetics of *Thiobacillus ferrooxidans* in batch cultures, containing prills of elementary sulphur as the sole source of energy, was studied by measuring the incorporation of radioactive phosphorus in free and adsorbed bacteria. The data obtained indicated an initial experimental growth of the attached bacteria until saturation of the susceptible surface was reached followed which a linear release of free bacteria due to successive replication of a constant number of adsorbed bacteria (Espejo and Romero, 1987).

Blake and Shute (2000) gave an account of respiratory enzymes of *Thiobacillus ferrooxidans* in which a soluble blue copper protein rusticyanin was reported during the reaction. The growth in the electric field was checked by Natrajan (1991) for which DC current was used. It was found that application of positive potentials up to +1000 mV in an acid bioleaching medium to be detrimental to bacterial activity, while the impression of negative potentials enhanced both their activity and growth through electrochemical regeneration of ferrous ions and an increase in biomass. The growth of *Thiobacillus ferrooxidans* was correlated with the dissolution of sulphur particles (Shrihari et al., 1992). In order to assess the biochemical activity many criteria were studied like Tyrosyl-t-RNA synthetase functions. The enzyme pyruvate dehydrogenase was characterized by Powels and Rawlings (1997). The heavy metal resistance of *Thiobacillus ferrooxidans* in different strains was studied by Leduc et al. (1997) which contain heavy metals like copper, nickel, uranium and thorium. Uranium and thorium were found more toxic in this study. The use of *Thiobacillus ferrooxidans* was done for the extraction of several metals either by direct method or indirect method. The biosorption of Cu was demonstrated by Escobar and Godoy (2002) for drainage control in the mines, it was used at large scale. The proteomic analysis of *Thiobacillus ferrooxidans* showed that the exported Rhodonase like protein was induced during the growth in metal sulphides and different sulphur compounds (Ramirez et al., 2002).

From sewage sludge the metals like copper and zinc were characterized for tolerance. The tolerance was estimated upto 20 to 200 m mol 1^{-1} for copper and zinc respectively (Barreria et al., 2005). The synthetic sediments were treated with *Thiobacillus ferrooxidans* in order to overcome the contamination of nickel and cadmium (Kim et al., 2005).

Table 1: List of bioleaching fungi with their sources of metals

	Name of the Fungus	Sources of metal extraction	Extracted metals	Workers	Year
1	*Fusarium sp.*	Silicate nickel ore	Cr, Fe and Mg	Bosecker	1989
2	*Aspergillus A_3 and Penicillium. P_2*	Nickel ores	Ni and Co	Tzeferis	1994
3	*Penicillium notatum, P. simplicissimum, T. viride and A. niger.*	Red mud	Al	Vachon et al.	1994
4	*Aspergillus niger*	Metal polluted soil	Zn, Cd, Mg and Ca	Akhtar and Mohan	1995
5	*P. simplicissimum*	Industrial filter dust	Al and Ni	Muller et al.	1995
6	*Aspergillus niger*	Kaolins	Al	Mesquita et al.	1996
7	*Aspergillus niger*	Fly ash from municipal incineration	Al, Cu, Pb, Mn, Cr, Ni, Cd and Fe	Bosshard et al.	1996
8	*P. purpurogenum, A. niger and Rhodotorula rubra*	Spodumene	Li	Rezza et al.	1997
9	*Aspergillus niger*	Copper mining residue	Cu	Mulligan and Cloutier	2000
10	*Aspergillus niger (NRRL567)*	Low grade bauxite ore	Al and Fe	Rashid et al.	2001
11	*Rhizopus delemar*	wastewater	Cd and Cr	Tsekora and Petrov	2002
12	*Penicillium citrinum*	Manganese ore	Mn	Acharya et al.	2004
13	*Aspergillus niger*	Incinerator fly ash	Zn	Xu and Ting	2004
14	*Aspergillus and Rhizopus sp.*	wastewater	Co and Ni	Ahmed et al.	2005
15	*Aspergillus niger*	Petrolium refinery waste	Al, Mo and Ni.	Aung and Ting	2005
16	*A. niger, P. notatum*	low grade bauxite ore	Al, Fe, Si and Ti.	Ghorbani et al.	2007
17	*A. niger and P. notatum*	Red mud	Al, Si, Ti and Fe	Ghorbani et al.	2008
18	*Aspergillus niger*	Industrial effluent	Cr.	Narvekar and Vaidya	2008
19	*Sacchromyces cerevisae*	Industrial waste	Zn	Sarla et al.	2008
20	*Aspergillus niger*	Fly ash	Cd, Mn, Pb, Zn, Cr and Fe	Wang et al.	2008

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21	*Aspergillus foetidus*	Nickel laterite ore	Ni	Valix et al.	2009
22	*Aspergillus niger*	Incineration fly ash	Zn, Pb and Mn.	Yang et al.	2009
23	*P. janthinellum strain GXCR*	Low grade chalcopyrite	Cu and Fe	Zhou et al.	2009

Table 2: List of bioleaching bacteria with their sources of metals.

	Name of the bacteria	**Sources of metal extraction**	**Extracted metals**	**Workers**	**Year**
1	*Bacillus licheniformis*	Magnesite ore	Silica, Mg	Mohanty et al.	1992
2	*L. ferrooxidans*	Pyrite	Fe and Cu	Sand	1992
3	*Pseudomonas putida and Corynebacterium glutamicum*	Industrial filter dust	Cu, Zn, Au, Ni, U, Hg & Sb	Muller et al.	1995
4	*Bacillus polymyxa*	Bauxite ore	Fe and Ca	Anand et al.	1996
5	*T. ferrooxidans*	Municipal waste incinerator	Cu, Cd, Pb, Zn, Al, and Fe.	Mercier et al.	1999
6	*T. ferrooxidans*	Pentalandite	Ni and Co.	Cawlina et al.	2000
7	*Thiobacillus spp.*	Contaminated sediments	Cu, Zn, Pb, Ni, and Cr.	Chen and Lin	2000
8	*Thiobacillus ferrooxidans and T. thiooxidans*	Fly ash, municipal solid waste	Cd, Cu, Al, Fe, Zn, Cr and Ni.	Krebs et al.	2000
9	*Micrococcus luteus Pseudomonas pseudoalcaligenes*	Industrial effluent and municipal wastewater	Cu, Ni, Zn, lead, Cr	Leung et al.	2000
10	*Bacillus sp.*	kaolins	Fe and Al.	Styrikova and Styriak	2000
11	*Pseudomonas B-2 and D-5*	Industrial effluent (Battery manufacturing)	Pb	Ekundayo and Killham	2001
12	*Painibacillus polymyxa*	Bauxite ore	Fe and Ca	Vasan et al.	2001
13	*T. ferrooxidans*	Nickel Iron sulphide	Ni, Fe, Cu	Mason and Rice	2002
14	*A .ferrooxidans*	Paper factory wast	Fe, Cu and Zn.	Picher et al.	2002
15	*T. ferrooxidans*	Industrial waste	Al, Zn, and Fe	Solisio et al.	2002
16	*P. fluorescens, B. megaterium and Chromobacterium violaceum*	Nickel powder & electronic scrap	Ni, Fe, Al and Zn	Faramarzi et al.	2004
17	*Serratia marcescens*	Silicates minerals	Al, Fe and silicate	Hutchens et al.	2003

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18	*Pseudomonas sp.*	Coal pile runoff	As, Se, Cr, Zn, Cd, Ni, Pb, Fe and Al	Ibeanusi et al.	2003
19	*T. ferrooxidans*	Dewatered sludge	Cu, Cr, Zn, Al, Fe	Kim et al.	2003
20	*A. ferrooxidans*	Chalcopyrite	Cu	Rahman and Gul	2003
21	*T. ferrooxidans*	Sewage sludge	Cd, Zn, Pb, Cr, Cu, Mn and Ni	Tyagi et al.	2003
22	*Thiobacillus spp.*	Sewage sludge	Zn, Ni, lead, Cu and Cr.	Villar and Garcia	2003
23	*T. ferrooxidans*	Complex sulphide	Co, Zn and Fe.	Ghosh et al.	2004
24	*Bacillus circulans, B. mucilaginosus*	Low grade bauxite ore	Al and Fe	Groudev	2004
25	*T. ferrooxidans*	Zinc sulphide ore	Zn	Hossain et al.	2004
26	*P. fluorescens Shewanella putrefaciens and P. stutzeri*	Mine tailing	U, Cr, Zn, Mn, V, Th and Co	Kalinowski et al.	2004
27	*A. ferrooxidans*	Low grade sarchesmeh copper sulphide	Cu and Fe.	Kolahdoozan et al.	2004
28	*A. ferrooxidans*	Complex galena concentrate	As	Mokita et al.	2004
29	*Bacillus and A. thiooxidans*	Ocean nodules	Co, Cu and Ni	Mukherjee et al.	2004
30	*Bacillus megaterium bl7 and Bacillus mycoides bi2*	Sulphides and silicate concentrate.	Si and Fe.	Styrikova et al.	2004
31	*A. ferrooxidans, A. thiooxidans and Lept. Ferrooxidans*	Cu/Pb/Zn sulphide bulk concentrate	Cu, Zn, Pb and Fe	Dave and Tipre	2004
32	*Pseudomonas sp.*	Manganese ore	Mn, Fe	Fernandes et al.	2005
33	*T. thiooxidans and Aspergillus niger*	Fly ash from municipal waste	Zn, Ni, Cu, Cd, Cr, and As.	Ishigaki et al.	2005
34	*A. ferrooxidans*	Dewatered sludge	Zn, Cu, Cr.	Kim et al.	2005
35	*A. ferrooxidans*	Sarcheshmeh molybdenite concentrate	Re and Mo	Zamani et al.	2005
36	*T. ferrooxidans and T. thiooxidans*	Low grade ores	Cu, U and Au	Bosecker.	2006
37	*A. ferrooxidans and L. ferrooxidans*	Coal	Hg	Klasoon et al.	2006
38	*A. thiooxidans*	Contaminated soil	Cr, Cu, Pb, Zn	Kumar and	2006

			and Cd.	Nagendran	
39	*A. ferrooxidans Sulfobacillus*	Low grade complex zinc/lead ore	Zinc	Mousavi et al.	2006
40	*Pseudomonas sp. E. coli WS11*	Industrial waste	Cr, Cu, Zn, Cd and Ni	Ansari and Malik	2007
42	*A. ferrooxidans and A. caldus*	Anilite	Cu	Cheng and Hu	2007
43	*A. ferrooxidans and T. thiooxidans*	Black shale	Cu and Ag.	Grobelzki et al.	2007
44	*Acidianus brierleyi*	chalcopyrite	Cu	Meng et al.	2007
45	*Sulfobacillus thermosulfidooxidans*	Electronic scrap	Ni, Cu, Al, Zn, Pb and Sn	Ilyas et al.	2007
46	*Bacillus, cereus, B. Megaterium and B. pumilus*	Feldspar raw material	Al, Si, Ti, Mn and Mg	Styriakova	2007
47	*T. ferrooxidans*	Steel plant waste	Zn, Fe and Al	Bayat et al.	2008
48	*P. fluorescens Chromobacterium violaceum, and P. plecoglossicida*	Solid waste material	Ag, Au and Pt	Brandl et al.	2008
49	*A. ferrooxidans and A.caldus*	Chalcopyrite	Cu and Fe	Chen et al.	2008
50	*A. ferrooxidans and A. thiooxidans*	Flotation concentrate of sphalerite	Zn	Chang et al.	2008
51	*A. ferrooxidans*	Spent lithium ion secondary batteries	Co and Li	Mishra et al.	2008
52	*A. ferrooxidans SRHGM1*	Low grade refractory sulphide gold ore 253	Fe and As	Dave and Tipre	2008
53	*A. ferrooxidans and T. thiooxidans*	Contaminated sand	Cr, Ni and Zn.	Gema et al.	2008
54	*P. aeruginosa*	Petrolium contaminated sites	Mn, Zn, Cr and Cu.	Maria et al.	2008
55	*Pseudomonas sp.*	Industrial waste	Cr (VI)	Srivastava et al.	2008
56	*A. ferrooxidans*	Fly ash & alkaline mine tailings	Cr, Cu, Ni, Zn, Al and Fe	Zhang et al.	2008
57	*Bacillus sp., Pseudomonas sp and Sporosarcina sp.*	Pb/Zn smelting slag	Al, As, Cu, Mn, Fe, Zn and Pb	Cheng et al.	2009
58	*Burkholderia caribensis FEGLO3*	Iron ore	Fe and P	Delvasto et al.	2009
59	*A. ferrooxidans, L. ferrooxidans and Sulfobacillus thermotolearans*	Low grade balck schist ore	Ni, Zn, Cu and Co.	Halinen et al.	2009
60	*Bacillus subtilis-38*	Contaminated soil	Cd	Jian et al.	2009
61	*A.ferrooxidans*	Chalcopyrite & Bornite	Cu	Wang et al.	2009

The strains of Pseudomonas are promising and playing role in the bioleaching process of metals due to their great adaptability from industrial wastewater were reported in maximum number which indicates high metal resistance (Malik and Jaiswal, 2000). The species showing metal resistence were identified Pseudomonas denitricans, Pseudomonas stutzeri, **Pseudomonas fluorescens**, Pseudomonas putida (Ottow and Glathe, 1970). Morphological and chemical changes in the attached cells of Pseudomonas aeruginosa as primary biofilms developed on aluminum and CaF_2 plates were studied by Cheung et al. (2006). A unique cellular feature similar in morphology to pili was observed on the surface of Pseudomonas aeruginosa adhering on aluminum but not on CaF_2. Aluminum, an environmental toxin, is known to have a negative impart on various biological systems. However some microbes have indicated mechanisms to combat the toxic influence of this trivalent metal. ***Pseudomonas fluorescens*** showed citrate synthesis mechanism to overcome the exposed aluminum toxicity (Mailloux et al., 2008). The adaptive and cross resistance to cadmium (II) and Zinc(II) by Pseudomonas aeruginosa BC 15 was studied by Raja et al., (2008). The study on metabolism of aluminum citrate and biosynthesis of oxalic acid in ***Pseudomonas fluorescens*** indicated that these metabolites were synthesized by it in aluminum stress condition (Apparna et al., 2003).

MECHANISM OF METAL BIOEXTRACTION

The mechanism by which the metals are extracted from the sources i.e. ores and other polluted sites of sewage and industrial effluent dumps. Biosorption is a mechanism of microbes in which the metals are accumulated on the surface of microbes some prokaryotic (Bacteria and Archaea) and eukaryotic (algae and fungi) microorganism can produce or excrete extracellular polymeric substances such as polysaccharides, glucoprotein, lipopolysaccharide, sulfate peptides etc. These substances posses a substantial quantity of anion functional groups which can adsorb metal ions. References published on metal biosorption with EPS mainly focus on the bacteria, *Bacillus megaterium, Acinobacter, Pseudomonas aeruginosa,* sulphate reducing bacteria cyanobacteria or activated sludge (Leiu et al, 2000; Wang et al., 2002). Dhami et al., (2002) worked on biosorption of American using various biomass of *Rhizopus* species. Wang and Chen, (2006) investigated the effect of EPS on Pb^{2+} removal by a polymorphic fungus *Aureobasidium pullulans*. The results showed that Pb^{2+} penetrated into the inner cellular part of the EPS extracted cells of *Aureobasidium pullulans*. The cell wall tends to be the first cellular structure to come in contact with metal ions, excluding possible existing extracellular layer mainly related to bacterial cells. Two basic mechanisms of metal uptake by cell wall are as follows stoichiometric interaction between functional groups of cell wall composition, including phosphate carboxyl, amine as well as phosphodiester and physicochemical inorganic deposition via adsorption or inorganic precipitation.

Now a day's complexion, ion exchange, adsorption, inorganic microprecipitation, oxidation and / or reduction have been proposed to explain metal uptake by organisms (Liu et al., 2003). When the extracellular concentration of metal ions was higher than that of intracellular, meal ions could penetrate into the cell across the cell walls and membrane of the biomass by free diffusion. Metal ions can also enter into the cell if the cell wall is disrupted by natural force (e.g. autolysis) or artificial force (mechanical force or alkali treatment etc.). The above process is independent of metabolism. To study this process various analytical techniques are used in metal biosorption which are atomic absorption spectrophotometry, infrared absorption spectroscopy or Fourier Transformed Infrared spectroscopy (IR or F TIR), scanning Electron microscopy, Transmission electron microscopy and X-ray photoelectron spectrum (XPS) (Lin et al., 2005). Brady et al. (1994) observed that the copper tolerant cell surface of S. cerevisae become converted, more spherical and smaller by SEM. Suh et al. (1998) confirmed the process of Pb^{2+} accumulation in S. cerevisae using a TEM technique. Lin et al. (2005) utilized multi techniques, including AAS. X-ray powder diffraction analysis (XRD) and XPS, to elucidate the mechanism of Au^{3+} removal by S. cerevisae. The results proved that Au^{3+} was reduced to Au^{0}

by an aldehyde group of the reducing sugars as the hydrolyzates of the polylsaccharides on the peptidoglycan layer of the yeast cells (Wang and Chen, 2006).

Ashaari et al., (2006) have given the account of sulphur oxidizing enzyme produced by bacteria isolated form effective microorganism active solution. The gram negative bacterium was found capable of growing autotrophically in sulphur oxidizing medium, containing 4 mm sodium thiosulphate that serves as an energy sources and electron donor. Sulphate ions were the expected ends product of sulphide oxidation catalyzed by extracellular sulphide oxidizing enzyme. Electron transport pathways of endogenous substrate(s) of *Thiobacillus ferrooxidans* was highlighted by Chen and Suzuki (2006) in which oxidation of endogenous substrate(s) of it with O_2 or Fe^{3+} as electron acceptor was studied in the presence of uncouples and electron transport inhibitors. A direct biohydrometallurgical extraction of Iron from ore based on reductive leaching of iron by anaerobic bacteria was studied by Eisele (2005) in which microorganisms were collected from an anaerobic bog where natural seepage of dissolved iron was observed. The enzyme activity of glutathione dependent sulfur dioxygenase was studied by Rohwerder et al., (2003). To identify the actual substrate of the enzyme elemental sulfur oxidation of the meso acidophilic *Acidithiobacillus thiooxidans* strain DSM -504 and K6, *Acidithiobacillus ferrooxidans* strain R1 and Acidiophilum DSM 700 was analyzed. Extraordinarily high specific sulfur dioxygenase activities upto 460 n mol min^{-1} were found in the crude extracts. All cell free systems oxidized elemental sulfur only via glutathione persulfide (GSSH) a non-enzymic reaction product from glutathione (GSH) and elemental sulfur. With bioleaching of copper from low grade ore using isolated bacteria and defined mixed cultures, the indirect mechanism of bioleaching was studied by Zilouei et al., (2003) in which *Thiobacillus ferrooxidans*, Thiobacillus thiooxidans, Leptospirillum ferrooxidans, Acidianum brierly and Sulfolobus acidocaldarus were used. The results obtained showed that the isolates were consuming acid and copper was extracted. The positive effect of the initial concentration of Fe^{2+}showed that major portion of copper extraction was via indirect method. Both aerobic and anaerobic nature of *Acidithiobacillus ferrooxidans* was demonstrated by Ohmura et al., (2002). In their studies they showed that the bacterium could chemolithotrophically grown not only on H_2O_2 under aerobic conditions but also on H_2/ Fe^{3+}, H_2 S^0 or S^0 / Fe^{3+} under anaerobic conditions. Anaerobic respiration using Fe^{3+} using Fe^{3+} or S^0 as an electron acceptor and H_2 or S^0 as an electron donor serves as primary energy source of bacterium.

Fly ash is the incombustible material matter which is left after all the organic components of the coal have been consumed during the process of coal combustion and is collected by means of mechanical electrostatic precipitators (Scheetz and Earle, 1998). Fly ash is composed of mainly silt-sized spherical amorphous ferr-aluminosilicate minerals and is generally characterized as having low permeability low bulk density as well as high specific surface area (Scheetz and Earle, 1998). The ash particle generally ranges in size from 0.5 to 200 μ (Querol et al., 1995). Fly ash particles are mainly composed of silicon (SiO_2), aluminum (Al_2O_3), iron (Fe_2O_3), titanium (TiO_2), and manganese (Mn2O3) (Scheetz and Earle, 1998).

The mechanism of bioleaching of coal fly ash (CFA) by Thiobacillus thiooxidans was studied by Brombacher et al. (1998)**.** The study of interactions between bacteria, metabolic products, CFA particles and leaching products were studied. It was demonstrated that bacterial growth and the amount of metals leached from the CFA were compared with biological and chemical interaction, which involve various components in this system. Kinetic model of CuS oxidation by *Thiobacillus ferrooxidans* and Thiobacillus thiooxidans bacteria was proposed by Domka (2001). The CuS oxidation with *Thiobacillus ferrooxidans* bacteria was found to be best described by the model of inhibition of the first order with respect to the substrate and product inhibitor, while the process with Thiobacillus thiooxidans and mixed cultures of these bacteria was best described by first order reaction with respect to the substrate. The dissolution activity

of two selected Bacillus cereus strains was investigated on three Kaolin samples contaminated with iron oxyhydroxides and mica. The experimental results showed that these bacteria are able to remove 43 % of free Fe occurring in Kaolin in amorphous form of oxyhydroxides and approximately 15 % of Fe bound in mica after one month of bioleaching. Bacterial leaching of metal sulphide by two indirect mechanisms via thiosulphate or via polysulphides and sulphur was studied by Schippers and Sand (1999) in which the acid insoluble metal sulphides FeS_2, MoS_2 and WS_2 were chemically attacked by iron (III), hexahydrate ions, generating thiosulfate, which was oxidized to sulphuric acid. Other metal sulphides were attacked by iron (III) ions and by protons, resulting in the formation of elemental sulphur via intermediary polysulphides. Sulphur is biooxidized to sulphuric acid. This explained leaching of metal sulphides by Thiobacillus thiooxidans, Acidiphilium urytoum JF-5 was found capable of coupling the reduction of Fe (III) to the oxidation of glucose. From chalcopyrite copper was extracted by *Thiobacillus ferrooxidans*. The influence of the mineral content in the leaching solution the pH value and refinement of chalcopyrite on the subsequent stages of leaching was established. The process was described by the 1st order reaction inhibited by one other product (Cu^{2+}) (Nowaczyk et al., 1998; Lorenzo et al., 1997). Bioleaching of a Spanish uranium ore in shaker and columns using natural pure and mixed cultures has been studied by Munoz et al., (1993). Different sources were used for the metal extraction such as only sulphur for *Thiobacillus ferrooxidans* (Sugio et.al., 1990) lead mattes (Ballester et.al., 1989), Bauxite (Ghorbani, 2008), Mangnese ores (Belyi et al., 2006), pyrite (Edwards et al., 1998). pH was found as limiting factor in the ferric iron reduction by *Thiobacillus ferrooxidans*. At extremely low pH values it was studied by Sand (1992). It was seemed to be inhibited at pH 1.3 and at a pH 1.8 the ferrous iron was deoxidized at once. Using waste water and contaminates the mechanism of metal extraction was elaborated out of which biosorption was found as better mechanism for metal leaching (Gupta et al., 2000), but the reduction of ions was also seen in case of waste water treatment containing hexavalent chromium which was reduced to trivalent by Micrococcus tetragon, Bacillus, Microbacterium and Spirillum (Yan-bin et al., 2005).

METHODS OF ANALYSIS

Analysis of extracted metals or dissolved metals is an important task of bioleaching of metals. The methods available are based mostly on spectrophotometry. Through molecular absorption spectroscopy determination of iron can be done using 1, 10-phenoanthroline (Demirhan and Fikriye, 2007). For trace iron in the fresh water the spectrophotometric methods have been suggested (Ivsic and Tamhina, 2003; Kawakubo et al., 2004). An ultraviolet spectrophotometric method for the determination of pyrite and ferrous ion oxidation is also available (Basaran and Tuovinen, 1986). The method of Atomic Absorption Spectrophotometry is popular in estimating the metals (Acharya et al., 2004).

For the analysis of aluminum many methods are given by different workers. Aluminum can be detected from the zirconium and titanium powders by spectrophotometry (George, 1968). Chrome azurol was used for the detection of micro amounts of aluminum in Iron and steel (Hosoya et al., 1961). Flow injection analysis for trace aluminum with on line preconcentration and spectrophotometric detection was done by Kobayashi et al. (1994). Using solid phase extraction spectrophotometrically aluminum could be determined (Ferreira et al., 1998) Nadszhafoya et al. (2005) have developed test paper for the determination of aluminum in solution. The test paper was made up of chrome azurol S. The spectrophotometric method with m-carboxyphenylfurane was developed for determination of aluminum (Kamino et al., 2005). The aluminum from natural wastes and industrial waste can be analyzed by sequential injection methodology followed by direct and kinetic spectrophotometric analysis (Mesquite and Raquel, 2008). Simultaneously iron and aluminum can be estimated by differential kinetic spectrophotometric method and chemometrics (Yonginian et al., 2007). A novel approach to

non segmented flow analysis was developed for aluminum analysis in river wastes (Lawes and Wong, 1991).

FACTORS AFFECTING BIOLEACHING OF METALS

During bioleaching of metals the process is enhanced or decreased in rate due to different limiting factors. Temperature is important factor which affect bioleaching. The thermotolerence of ferrous oxidizing bacterial isolates was studied for chalcopyrite bioleaching. Three ferrous oxidizing, acidophilic bacterial isolates oxidized Fe^{2+} when growing on yeast extract. One isolate but not the other two grew on ferrous sulphate as the only energy source. The isolates did not grow on pyrite, sulphur or thiosulphate, but they grew on chalcopyrite and copper. They were mesophiles but could be adapted to grow at 38 ^{0}C and 45 ^{0}C (Lorenzo et al., 1997). At different temperatures a kinetic study of the depyritization of oil shale HCl Kerogn concentrate by *Thiobacillus ferrooxidans* was done by Vrvic et al. (2003). The oxidation of sulphate by Thiobacillus thiooxidans was studied at various pH values with changing concentration of potassium sulphate. The sulphate oxidation by cells was inhibited at high sulphate concentrations, particularly at low pH values.

The effect of various ions, pH and osmotic pressure on oxidation of elemental sulphur by Thiobacillus thiooxidans was studied by Suzuki et al. (1999). The oxidation of elemental sulfur by Thiobacillus thiooxidans was studied at pH 2.3, 4.5 and 7.0 in the presence of different concentrations of various anions (sulphur, phosphate, chloride, nitrate, fluoride etc.) and cations (K, Na, Li, Rb and Cs). The results agreed with the expected response of the acidophilic bacterium to change neutralization of colloids by ions, pH dependent membrane permeability of ions and osmotic pressure. Effect of sludge dissolved organic matter on oxidation of ferrous iron and sulfur by *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* was studied by Fang and Zhou, (2005). The effects of bacterial leaching on metal partitioning in sewage sludge were studied by Lombardi et al. (2006). The partition of Mn, Al, Zn, Cu and Ti ions in municipal sewage sludge was investigated before and after bioleaching process affected by A. ferrooxidans and A. thiooxidans. Oxidation-reduction potential increase and pH decrease obtained as a result of bacterial activity. Apart from the normal growth of *Thiobacillus ferrooxidans*, it is also isolated from heavy metal contaminated environments such as soil and sewage sludge and is known to be useful in bioremediation for these environments as it has shown that T. ferrooxidans did not respond to dibutyl phthalate as far as oxidizing of ferrous, ability is concerned although changes in growth medium were accompanied in the protein expression profiles of periplasmic fraction and remarkable changes in ultra-structure of the cell (Wang et al., 2009).

BIOREMEDIATION OF METAL POLLUTION

Bioremediation is derived from the combination of biochemical-microbiological and ecological processes which can be summarized as under. The relationship of comparative biochemistry apply both in the axenic culture of microorganisms and in field soil. The microbial growth requirements are the same whether in the laboratory culture or in the field.

The limitations resulting from ecological interaction including the need to accommodate the pressure of other microbes on the microbes (Mussarat and Zaidi, 2005). Microorganisms can physically remove heavy metals and radionuclide from solution through either bioaccumulation or biosorption. Bioaccumulation is the retention and concentration of a substance by an organism. In this process, the metals are transported from outside the microbial cell through cell membrane into cytoplasm, where the metal sequestered and immobilized. However in biosorption the positively charged metal ions are sequestered primarily through adsorption to the negative ionic groups on cell surfaces, such as capsules or slime layers. Binding sites on microbial cell surfaces usually are carboxyl residues, phosphate residues, SH groups or hydroxyl groups. The amount of metal biosorbed to the exterior of bacterial cells often exceeds

the amount predicted, depending upon the charge density on the cell surface. The charged functional groups serve as nucleation sites for deposition of various metal bearing precipitates. Many studies have demonstrated the efficiency of metal removal by microbial biomass in a range of reactor formats and under varying physical and chemical conditions (Rashmi et al., 2007).

Heavy metal pollution of soil is a significant environmental problem and has its negative impact on human health and agriculture. Rhizosphere is an important interface of soil and plant which plays a significant role in phytoremediation of contaminated soil by heavy metals. In this process microbial population are known to affect heavy metal mobility and availability to the plant through release of chelating agents, acidification, phosphate solubilization and redox changes, and therefore, have potential to enhance phytoremediation processes. Phytoremediation strategies with appropriate heavy meal adopted Rhizobacteria have received more and more attention. Rhizobacteria have been shown to possess several traits that can alter heavy metal bioavailability through the release of chelating substances, acidification of the microenvironment and by influencing changes is redox potential (McGrath et al., 1988). Remediation of heavy metal polluted sediment in the solid bed was studied with special reference to comparison of abiotic and microbial leaching by Loser et al. (2006). In this study elemental sulphur added to the sediment was oxidized to sulphuric acid which solubilizes heavy metals. Here the percolation flow and the solid-bed height did not affect the rate of metal solubilization.

Similarly, the same bacterium was used for decontamination of fly ash and used lime from municipal waste (Mercier et al., 1999). The mercury was removed from mercury contaminated sediments using a combined method of chemical leaching and volatilization of mercury by bacteria (Nakamura et al., 1999). For the treatment of heavy metal containing effluents the microbial biofilms were developed on various surfaces such as grand glass and roughened polyester dream beds (Meyer and Wallis, 1997).

Along with purification of the effluents the metal extraction was carried out from industrial effluents in variable quantities. The concentrations of twelve different heavy metals in the waste were studied by Mehta and Churi (1999), in which As, Cd, Pb, Cu, Zn, Mo, V, Ni, Hg, Se, Cr and Mn were recorded. The metals like Zn, Ni, Cr and Cu containing sludge from galvanic industries was studied by Brombacher et al. (1998). The leaching yield was found in Zn was 99 % while for Ni, Cu and Cr were 96, 81 and 71 % respectively. Bioleaching of Zinc and Iron form steel plant waste was done by Bayat et al. (2008) using *Thiobacillus ferrooxidans*. The results showed that pulp solids concentration, slurry pH and solid particle size were all important parameters in the bacterial leaching process. Transformation of red mud from aluminum industry into a coagulant for waste water treatment was studied by Poulin et al. (2008). Removal of Cr, Pb and Cu ions from industrial waste waters was carried out by Staphylococcus saprophyticus (Ilhan et al., 2004). The bacterial species Pseudomonas pseudoalkaligens and Micrococcus luteus were found capable of removing significant amount of copper and lead. (Leung et al., 2000). The organic material is also served as a good source for leaching of the metals. Poultry liter is typically land applied as a means of disposal to be accumulated from poultry wastes. (Pirani et al., 2006). The extraction of Uranium from shale wine failings was studied by Kalinowski et al. (2004). **Pseudomonas fluorescens**, Shewanella putrefaciens and Pseudomonas stutzeri were reported to extract Uranium along with cobalt, Zinc, Manganese and Vanadium. ***Pseudomonas fluorescens*** was found to be better for Uranium extraction. Another species of Pseudomonas putida was found able for removal of Cu from industrial wastes (Wang and Brusseau, 1993). The anaerobically digested waste water sludge was also used for heavy metal extraction by *Thiobacillus ferrooxidans* at different pH (Wang et al., 2002).

GENETICS OF METAL BIOEXTRACTION

The use of acidophilic, chemolithotrophic microorganisms capable of oxidizing iron and sulphur in industrial processes to recover metals from minerals ores containing copper, gold and uranium, is well established biotechnology with distinctive advantages over traditional mining. A consortium of different organisms participates in the oxidative reaction resulting in the extraction of dissolved metal values from ores. Considerable efforts has been spent in the last years to understand the biochemistry of iron and sulphur compound oxidation, bacteria mineral interaction (Chemotoxis, quorum sensing, adhesion and biofilm formation) and several adaptive responses allowing the microorganisms to survive in a bioleaching environment. All of these are considered as key phenomena for understanding the process of mining. The use of genomics, metagenomics and high throughput proteomics to study the global regulatory responses that the biomining community uses to adapt to their changing environment is just beginning to average in the last years. Initial studies on the isolation, cleaving and sequencing of mostly individual chromosomal genes and plasmids of *Thiobacillus ferrooxidans* and expression of some of them in Escherichia coli to generate functional proteins have been reviewed (Rawlings, 2002). All these efforts have contributed greatly to the identification and isolation of various components of the iron oxidation system. Recently Rawlings (2005) reported the plasmids PTF-FC2 and PTC-F-14 isolated from two related bacteria, *Thiobacillus ferrooxidans* and *Acidithiobacillus* caldus respectively. These two plasmids encode for several genes related with their replication, addition and mobilization and belong to the Inc like plasmid family and must have originated from the some ancestral plasmid, however, only two of the five mobilization genes have remained highly conserved, whereas the other three genes appear to have evolved such that each plasmid is mobilized most efficiently by a different self transmissible plasmid (Rawlings and Johnson, 2007). These plasmids are very important, since they may help in the development of an efficient conjugational system for genetic studies of these microorganisms (Valenzuela et al., 2006). Plasmid encoded heavy metal resistance in Pseudomonas sp. was worked out by Unldi et al. (2003). The character of heavy metal resistance on plasmid was confirmed by curing a plasmid with Ethidium bromide and by transforming plasmid DNA into competent Escherichia coli AB 3505. Malik et al. (2002) studied two bacterial isolates from agriculture and industrial soils and resistance to metals like Cu, Cr, Pb, Cd, Hg, Zn and Ni for each isolate was determined. The highest MICs observed were 100 µg /ml for Hg. 800 µg/ml for Ni and 1600 µg/ml for other metals. On a percentage basis 91.4 % of the total bacterial isolates from industrial soil were found to harbour plasmids whereas 40 % of the isolates from agricultural soil contained plasmids. Similar work was confirmed with *Thiobacillus ferrooxidans* (Chisholm et al., 1998). Isolation, sequence analysis and comparison of two plasmids (28 and 29 kb) from the biomining bacterium, Leptosprillum ferrooxidans ATCC 49879 was worked out by Coram et al. (2005). A cluster of five genes, proposed to be involved in the formation of extracellular polysaccharide (EPS) precursors via the Leloir pathway, have been identified in the acidophilic autotrophic *Acidithiobacillus ferrooxidans*. The order of genes is Lux-A, gal E, gal K, pgm-gul M, encoding a Lux-A like protein, UDP-glucose 4-epimerase, glutokinase, phosphoglucomutase and galactose mutarotase respectively. The Arsenic resistance gene of *Thiobacillus ferrooxidans* was reported on chromosomal DNA. These were cloned and sequenced. Homogenous of four arsenic resistance genes, ars B, ars C, ars H and a putative ars R gene were identified. The *Thiobacillus ferrooxidans* ars B (arsenite export) and ars C (arsenite reductase) gene products were functional when they were cloned in an Escherichia coli ars deletion mutant and conferred increased resistance to arsenite and antimony. Therefore despite those ars genes originated from an obligately acidophilic bacterium, they were functional in E.coli. Although *Thiobacillus ferrooxidans* is gram negative, its Ars C was more closely related to the Ars C molecules of gram positive bacteria. (Butcher et al., 2000). The genetic transformation of obigately chemolithotrophic Thiobacilli was studied by Yankofsky et al. (1983). The transformation of

Thiobacillus thioparus auxotroph to prototrophy was obtained at frequencies of up to 10^{-2} when proliferating cell populations were exposed to chromosomal DNA from a nutritionally independent strain of the same bacterium (Kusano et al., 1992).

Plasmid and transposon transfer to *Thiobacillus ferrooxidans* was studied by Peng et al. (1994). The broad host range Inc P plasmids RP4. R68.45, RPI: Tn 501 and PUB 307 were transferred to *Thiobacillus ferrooxidans* from *Escherichia coli* by conjugation. A genetic marker of kanamycin resistance was experienced in *Thiobacillus ferrooxidans*. The transfer of plasmids from *Escherichia coli*, *Thiobacillus ferrooxidans* secured in the absence of energy sources for both donor and recipient. Transformation of *Escherichia coli* with a large plasmid of *Acidiphilium multivorurm* AIU 301 encoding Arsenic Resistance was studied by Suzuki et al. (1997). This bacterium harboured a number of plasmids with different molecular sizes. A plasmid of 56 kbp, named p KW301 was isolated from A. multivorurn AIU301. When pkw301 was transferred into *E. coli* JM 10a by electroporation, an *E. coli* transformants carrying pkw301 exhibited resistance to sodium arsenite, sodium arsenate and mercuric chloride. The poison antidote stability system for the broad range *Thiobacillus ferrooxidans* plasmid PTF FC2 was also studied by Smith and Rawlings (1997).

The genetics of metal leaching microbes can also be studied by considering genomic DNA characters pH gradient induced heterogeneity of Fe (III) reducing microorganisms in coal mining associated sediment. In this study the impact of pH on Fe (III) reducing activities in both of sediment zones Upper acidic zone (pH 3) with large amounts of reactive iron and a deeper slightly acidic zone (pH 5.5) with smaller amounts of iron were investigated and molecular analysis elucidated the sediment microbial diversity were performed. PCR products specific for species related to *Acidiphilium* revealed that there were higher numbers of phylotypes related to culture Acidiphilium species in zone III than in zone I. From the PCR products obtained for bioleaching associated bacteria, only one phylotype with a level of similarity to *Thiobacillus ferrooxidans* of 99 % was obtained. (Blothe et al., 2008). Structural analysis of 16s rRNA gene sequence from strain of *Thiobacillus ferrooxidans* was performed by Peng et al. (2005). Phylogenetic heterogeneity of the species *Thiobacillus ferrooxidans* was studied by Karavaiko et al. (2003). Genomic organization of the *Thiobacillus ferrooxidans* ATCC 21834 was studied by Irazabal et al. (1997). The genomic organization of the acidophilic *Thiobacillus ferrooxidans* ATCC 21834 has been studied by pulsed field gel electrophoresis (PFGE). Fournier et al., (1998) studied the bacterial populations involved in the metal bioleaching of wastewater sludge. They were able to enumerate more than 20 different bands, revealing a bacterial diversity undetected by cultivation techniques. Regulation of the expression of the Thiobacillus ferrooxidans rus operon encoding two cytochrome C, a cytochrome oxidase and rusticyanin was studied by Yarzabal et al. (2004) by Northern hybridization, real time PCR and reverse transcription them in sulfur grown cells. Phenotypic switching of *Thiobacillus ferrooxidans* was studied by Schrader and Holmes (1988) for these two solid medium formations 100:10 and 10:10 were developed for the growth of *Thiobacillus ferrooxidans*. The new media contain a mixture of both ferrous iron and thiosulfate as available energy sources, permitting thee detection of colony morphology variants that arise spontaneously in a wild type population. Several morphological and physiological characteristics for clones of *Thiobacillus ferrooxidans* variants termed LSC for large spreading colony LSC variants lack the ability to oxidize iron but retain the capacity to utilized thiosulfate or tetrathionate as energy sources. The molecular cloning of the gene encoding *Thiobacillus ferrooxidans* Fe (II) oxidase was done by Kusano et al. (1992) by determining amino terminal sequence of *Thiobacillus ferrooxidans* Fe (II) oxidase and the iro gene that encodes this enzyme was cloned using degenerate oligonucleotides as a probe. The technique, fluorescent in situ hybridization was used for detecting catalysis of sludge bioleaching done by acidophilic microbial communities (Bouchez et al., 2006).

GENETIC IMPROVEMENT OF BIOLEACHING MICROBES

Mutation studies on metal bioleaching bacteria are also remarkable. The physical mutagen ultraviolet light was widely used to induce mutation. Bhattacharya and Paul (2001) indicated that Caffeine and Acriflavine treatment enhanced UV sensitivity in *Thiobacillus ferrooxidans* cells. With the increase in concentrations of these compounds, the extent of survival decreased. Similarly the inhibitory effects of caffeine and Acriflavine increased with the increase in dose of UV-irradiation. The cells irradiated with 10 s of UV exposure tended to become resistant to the inhibitory effects of caffeine and acriflavine, as evidenced by the fine course study of recovery. The cells appear to stage a dramatic recovery from UV damage in presence of caffeine (3 mg/ml) and acriflavine (20 mg/ml) over a period of 25-30 had 35-40 h respectively, when grown in the presence of energy sources. The crystal structures of the Met 148 Len and Ser 86 Asp mutants of Rusticyanin from *Thiobacillus ferrooxidans* was studied by Kanbi et al. (2002). Construction and characterization of a rec A mutant of *Thiobacillus ferrooxidans* by marker exchange mutagenesis was studied by Lieu et al. (2000). To knock out the Thiobacillus ferrooxidans rec A gene a mobilizable suicide plasmid carrying the ATCC 33020 rec A gene disrupted by a Kanamycin resistance gene was transferred from E. coli to Thiobacillus ferrooxidans ATCC 33020 by conjugation under the best conditions determined. These mutants exhibited a slightly reduced growth rate and an increased sensitivity to UV and γ irradiation compared to the wild type strain. A DNA region that complements on Escherichia coli cys G mutation in *Thiobacillus ferrooxidans* was studied by Suzuki et al. (1997). The gene responsible for the appearance of NADH dependent sulphate reductase activity was cloned into a vector plasmid pBR 322 to give a 5.7 kb hybrid plasmid, PTHS1, which contains a 1.3 kb DNA fragment of *Thiobacillus ferrooxidans* AP 19.3. Ochman et al. (2000) demonstrated that horizontal gene transfer produces extremely dynamic genomes in which substantial amount of DNA are introduced into and deleted from the chromosome. These lateral transfers have effectively changed the ecological and pathogenic character of bacterial species.

Proteomics of *Thiobacillus ferrooxidans* was also studied by Ramirez et al. (2004). Differential protein expression during growth of *Thiobacillus ferrooxidans* on ferrous iron, sulfur compounds or metal sulphides was studied. For that a set of proteins that changed their levels of synthesis during growth of *Thiobacillus ferrooxidans* ATCC 19859 on metal sulphides thiosulfate, elemental sulfur and ferrous iron were characterized by using two dimensional polyacrylamide gel electrophoresis N- terminal amino acid sequencing and mass spectrometry analysis of these proteins allowed their identification and the localization of the corresponding genes in the available genomic sequence of *Thiobacillus ferrooxidans* ATCC 23270. The results showed that there is a separation of iron and sulfur utilization pathways. Rusticyanin in addition to being highly expressed on ferrous iron was also newly synthesized, as determined by metabolic labeling although at lower levels, during growth on sulfur compounds and iron free metal sulphides. During growth on metal sulphides containing iron, such as pyrite and chalcopyrite, both proteins up regulated on ferrous iron and those up regulated on sulfur compounds were synthesized, indicating that the two energy generating pathways are induced simultaneously depending on the kind and cancellation of oxidizable substrates available.

MATERIALS AND METHODS

A] PHYSICO CHEMICAL ANALYSIS OF METAL SOURCES:

1] Sample Collection and Physico – Chemical Analysis of Bauxite Ore:

The bauxite ore sample was collected from the bauxite mines at Radhanagari, Kolhapur. The sample was collected (0.5 – 0.8 kg) from different sites and various depths of mines and immediately sealed in the sterile polythene bags and brought to the laboratory. The collected sample was crushed and ground to fine particles and stored at 4^0C in refrigerator until use. The collected bauxite ore was subjected to analyze the microbiological (microflora) and Physico chemical characters such as colour, pH, Redox potential, conductivity, salinity, and mineralogical characterization.

2] Sample Collection and Physico – Chemical Analysis of Fly Ash:

The fly ash used in the experiment was collected from Parli Thermal Power station premises at moist places away from dumping sites. 1-2 kg fly ash was collected in air tight polyethylene bags sterilized with alcohol. The collected ash was washed with water to remove water soluble compounds and dried on hot plate. The collected fly ash was subjected to analyze the microbiological (microflora) and Physico-chemical characters such as colour of the ash, pH, Redox potential, conductivity, salinity and mineralogical characterization were analyzed as per the method of Black et al. (1965). The physical properties of the fly ash bulk density, maximum water holding capacity, porosity were analyzed as per the methods of soil analysis.

3] Mineralogical and Elemental Analysis of Bauxite Ore:

X-ray diffraction technique (XRD) was used to determine the mineralogical composition of bauxite ore using Rigaku Rint 300 series Diffractometer. Finely ground bauxite ore sample was analyzed with a glass slide mount using Cu.Ku radiation. A wide range goniometer was used. The Diffractometer was equipped with curved graphite monochromator and θ compensating site.

4] Mineralogical and Elemental Analysis of Fly Ash:

X-ray diffraction technique (XRD) was used to determine the mineralogical composition of fly ash using Rigaku Rint 300series Diffractometer. Finely ground fly ash sample was analyzed with a glass slide mount using Cu.Ku radiation. A wide range goniometer was used. The Diffractometer was equipped with curved graphite monochromator and θ compensating site.

5] SEM and EDX Analysis of Bauxite Ore:

The morphological changes in the surfaces of individual minerals were investigated by SEM (scanning electron microscopy) and the changes of chemical composition and element present on surface of the ore sample by Energy Dispersive X-ray (EDAX) analysis. All mineral samples were coated with platinum and subsequently examined in a scanning electron microscope Jeole 3500.

6] SEM and EDX Analysis of Fly Ash:

The morphological changes in the surfaces of individual minerals were investigated by SEM (scanning electron microscopy) and the changes of chemical composition and element present on surface of the fly ash sample by Energy Dispersive X-ray (EDAX) analysis. Mineral samples were coated with platinum and subsequently examined in a scanning electron microscope Jeole 3500.

7] Chemical Analysis of Bauxite Ore:

Chemical leaching was performed according to method employed by Francis et al. (1999). Collected sample of bauxite ore was crushed and ground to fine particles and used for acid leaching. For chemical analysis 2 gm of bauxite ore was digested in 10 ml concentrated H_2SO_4 at 100 ^{0}C. Cooled sample was kept away from water for 24 hours. Acid digested sample was

filtered through Whatmann filter paper no.41 and the filtrate was used for spectrophotometric detection of leached metals.

8] Chemical Analysis of Fly Ash:

To assess the metals present in coal fly ash according to method, developed by Francis et al. (1999) the collected ash was washed with water to remove water soluble compounds and dried in oven at 100 ^{0}C temperature. 2 gm of oven dried fly ash was mixed with 6 ml of nitric acid (HNO_3) and kept as it is for 24 hours. After 24 hours sample was filtered. Filtrate was used for spectrophotometric analysis of aluminum iron and manganese after appropriate dilution.

B) MICROBIOLOGICAL ANALYSIS OF BAUXIE ORE:

1] Isolation of Indigenous Fungi **from Bauxite Ore:**

From the collected soil samples, one gm bauxite ore was ground to fine powder and suspended in 100 ml distilled water. The solution was stirred mixed well to separate fungal spores from bauxite ore particles. One ml suspension was taken from mixture and diluted to 10^3 times and was spreaded on the modified Czapec Dox medium [composition in g/l – glucose-10, KNO_3 - 2.0 , KH_2PO_4 - 1.0 , KCl – 0.01 $MgSO_4.7H_2O$- 0.5 $FeSO_4$ -0.01 Agar-15.00, $CuSO_4.7H_2O$- 0.01, $ZnSO_4.7H_2O$ -0.01 and distilled water -1000 ml] (Krishna et. al., 2005).

Mineral salt solution was sterilized by autoclaving at 121 ^{0}C for 15 minutes and glucose solution was sterilized at same temperature for about 5 minute. The pH of the medium was adjusted to 6.5.

Inoculated plates were incubated for 4 days at 30 ± 2 ^{0}C at room temperature. Well grown fungal colonies were counted, purified and stored at 4 ^{0}C. Inoculated slants were used for further studies. All the isolates of fungal strains were purified by repeated culturing and sub culturing and single colony of each fungus was isolated on Rose Bengal Agar medium [Composition g/l Dextrose-10.0, Peptone-5.0, $MgSO_4.7H_2O$ – 1.0, $FeSO_4$ -0.5, Rose Bengal - 0.03, Agar-agar-20 and Distilled water-1000ml] (Martin, 1950). The frequency of bacteria and actinomycetes was also calculated by spreading the serially diluted samples on nutrient agar medium and Jensen's agar medium respectively.

C) ISOLATION AND CHARACTERIZATION OF METAL EXTRACTING MICROBES FROM BAUXITE ORE:

1] Isolation of *Thiobacillus ferrooxidans*:

For isolation of bioleaching microorganisms ore was collected from Radhanagari bauxite mine at various depths. Collected samples were transferred in the pre sterilized polyethylene bags and stored at 4 ^{0}C in refrigerator until use. From the collected soil samples 1 gm of bauxite ore was ground to fine powder and mixed in 100 ml of 9K medium [composition g/l ammonium sulphate $(NH_4)_2SO_4$ - 3.0, magnesium sulphate $MgSO_47H_2O$ -0.5, potassium hydrogen phosphate K_2HPO_4 - 0.5, potassium chloride KCl- 0.1, calcium nitrate $Ca(NO_3)_2$ -0.01, ferrous sulphate $FeSO_4.7H_2O$- 21.00] (Silverman and Lundgren, 1959). The pH of the medium was adjusted to 2.0 with 10N H_2SO_4. The culture in 9k medium was incubated for 1 to 4 weeks until growth was observed microscopically or until a chemical change occurred in the medium compared with an un-inoculated control.

The inoculated flasks were incubated at 32 ± 2 ^{0}C for 1 to 3 weeks at 140 rpm constant shaking condition. The presence of iron oxidizing bacteria in the liquid 9K medium was indicated by the formation of a characteristics ferric precipitation and orange coloring of medium. A serial dilution of culture was spreaded on 9K solid medium. The inoculated plates were incubated at 32 ± 2 ^{0}C for 10 to 20 days residence time. To observe the colony size, shape, colour and other morphological features. Single colony was picked from the plate by using a sterile inoculation loop and streaked on newly made plates.

2] Isolation of *Pseudomonas fluorescens*:
To isolate efficient indigenous bioleaching microbes from bauxite ore sample was collected and stored in refrigerator at 4 ^{0}C. To isolate dominant heterotrophic microbes LB broth was used. To isolate the indigenous microbes 2 gm of bauxite ore was inoculated with 40 ml of Luria Bertani broth in aseptic conditions. Inoculated flasks were incubated at 32 ± 2 ^{0}C for 4 to 6 days at 140 rpm constant shaking conditions.

The development of turbidity in the medium was assumed to be due to microbial growth. Serial diluted turbid sample was streaked on solid LB agar medium. [Composition g/l trypton -10.0, yeast extract – 5.0, NaCl-5.0, Agar -15] and incubated for 48 hours. After 48 hours, grown dominant colonies were selected for further purification and biochemical identification.

3] Morphology and Cultural Characteristics:
A Gram staining of the bacterial smears was done according to the method developed by Vincent (1970). Motility of the isolates was observed by hanging drop technique. Cultural characteristics of the isolates were studied on 9K agar plates for *Thiobacillus ferrooxidans* and Kings Medium for ***Pseudomonas fluorescens*** incubated at 36 ± 2 ^{0}C and 32 ± 2 ^{0}C respectively. The characters considered were colony size, shape, colour, margin, elevation, opacity, pigmentation and consistency.

4] Biochemical Properties

A) Catalase Test
The isolates were streaked on Luria Bertani agar medium plates and incubated for 24 hour at 32 ± 2 ^{0}C temperature. Hydrogen peroxide [H_2O_2] was poured on the culture slants and checked for effervescence.

B) Nitrate Reduction
The isolated Cultures were grown in 5 ml peptone water medium for 24 hours at 32 ± 2 ^{0}C temperature. 0.5 ml of Griess-Ilosway's reagent A, sulphanilic acid was added followed by 0.5 ml Griess-Ilosway's reagent B, α-napthyl amine and the colour change was noted. The nitrate when reacted with Griess-Ilosway's reagent gives red coloration. Un-reacted nitrate showed effervescence on addition of zinc dust.

Griess-Ilosway's reagent

Solution A - Sulphanilic acid- 8.0, 5 N acetic acid- 1000 ml

Solution B - Dimethyl- alpha-napthylamine- 5.0 gm,

5 N acetic acid-1000 ml

C) Gelatinase Test:
Isolates were inoculated on gelatin medium (Gelatin agar- Gelatin 4 g, glucose – 0.05 g, KH_2PO_4- 0.5 g, K_2HPO_4- 1.5 g, Nutrient agar (melted) – 1000 ml.

The phosphates, gelatin and glucose were dissolved in melted nutrient agar, slowly for even distribution of gelatin) and incubated at room temperature for 7 days. The tubes were placed in ice bath for 30 minutes and checked for liquifaction of gelatin each day. The plates with growth of bacteria were flooded with Frazier's solution (Mercuric chloride- 15 g, Hydrochloric acid- 20 ml, Distilled water- 100 ml). Gelatin hydrolysis indicated by clear zone around colony against opaque background.

D) Casein Hydrolysis:
The milk agar plates were inoculated with the isolates to check the protease activity. The casein hydrolytic colonies were identified which showed clear zone around the colony after incubation.

(Milk agar- Nutrient agar- 99 ml, skimmed milk- 1ml sterilized separately and mixed).

E) Oxidase Test:
The colonies were picked and streaked on filter paper, which was moistened with 1% (w/v) aqueous solution of tetra methyl–p-phenylene diamine dihydrochloride. In a positive reaction a purple coloration developed immediately within 10 seconds.

F) Motility
Motility of the isolates was studied by hanging drop technique and also by stab inoculating the cultures in Luria Bertani soft agar medium.

G) Production of Indole from Tryptophan:
Tryptone water (Tryptone- 1 g, sodium chloride- 0.5 g and distilled water- 100 ml) tubes were inoculated with isolates and incubated for 3 days. Indole production was tested with Kovac's reagent (Amyl or Isoamyl alcohol- 150 ml, p-dimethyl-aminobenzaldehyde- 10 g and Hydrochloric acid- 50 ml). A deep red colour indicated indole production.

H) Methyl Red Test
The bacterial isolates were inoculated in glucose phosphate broth (Peptone- 5 gm, glucose- 5 gm, K_2HPO_4- 5 gm and distilled water- 1000 ml). After incubation for 3 days, acid production was tested by adding methyl red solution (0.1-Methyl red, in 300 ml of 95 % ethanol made up to 500 ml with distilled water) red coloration indicated acid production.

I) Voges Proskuer Test:
Pseudomonas and Thiobacillus isolates were incubated in tubes containing glucose phosphate broth and were inoculated for 3 days. To observe results, Barritts modified reagent (Barritt, 1936) was used 0.5 ml of 6 % Napthol solution and 0.5 ml 16 % KOH were added to culture tubes. Development of red coloration indicated production of acetyl methyl carbinol, a neutral compound from glucose.

J) Effect of Aeration on Bacterial **Growth:**
The effect of oxygen and agitation in the medium at pH 4.5 for Thiobacillus isolates and 7.0 for Pseudomonas isolates was studied after incubation at 32 ± 2 ^{0}C temperature for 48 hours at 140 rpm in incubator shaker. Anaerobic condition was maintained with a 1cm layer of liquid Paraffin added to the top of the growth medium held in tubes and with no shaking.

K) Chemoorganotrophic Growth:
The glucose Thiobacillus medium was used to test for chemoorganotrophic growth contained no initial source of ferrous sulphate compounds. [composition g/l - Glucose -10 Ammonium sulphate [$(NH_4)_2SO_4$] -3.0, Magnesium sulphate [$MgSO^4.7H_2O$] -1.0, Potassium Di-hydrogen phosphate [KH_2PO_4]-0.5, Calcium dinitrate – [$Ca(NO_3)_2$] – 0.018 mg, Potassium Chloride [KCl] -0.10, Ferrous sulphate [$FeSO_4$] -0.01 mg, distilled water- 1000 ml, pH was adjusted to 5.5 with 1N H_2SO_4 In 1000ml distilled water].

One loopful from slant culture of each isolates was inoculated in to 100ml of the medium and incubated at 32 ± 2 ^{0}C temperature for 2- 4 days at 140 rpm in incubator shaker. Turbid culture was spreaded on glucose Thiobacillus agar medium and incubated at 32 ± 2 ^{0}C temperature for 2- 4 days.

L) Effect of Ph on Bacterial **Growth:**
Freshly prepared 9K broth and Kings medium with various pH ranges were inoculated with Thiobacillus and Pseudomonas and incubated for 48 hours at 32 ± 2 ^{0}C temperature with continuous 140 rpm shaking conditions. The pH of the medium was adjusted with 1 N H_2SO_4 and 1N NaOH. The growth was recorded in terms of relative turbidity.

M) Effect of Sodium Chloride Concentration on the Bacterial **Growth:**
To assess the deleterious effects of sodium chloride concentration on Thiobacillus and Pseudomonas isolates, 9K and Kings broth flasks were inoculated with both the isolates and incubated at 32 ± 2 and 36 ± 2 ^{0}C for 48 hours respectively. The agitation speed was 140 rpm and the growth of both the isolates was recorded in terms of relative turbidity.

N) Effect of Temperature on the Bacterial Growth:
To optimize the appropriate temperature for bacterial growth, the bacteria, Thiobacillus and Pseudomonas were inoculated in 9K and Kings broth tubes respectively. Both the isolates were incubated at different temperatures with continuous 140 rpm shaking condition. The growth at different temperature was recorded in terms of relative turbidity.

O) Ferrous Iron Oxidation in Bauxite Ore and Fly Ash by *Thiobacillus ferrooxidans*:
Ferrous iron oxidation by *Thiobacillus ferrooxidans* in presence of bauxite ore and fly ash was estimated by spectrophotometric method. Ferrous sulphate oxidation was monitored by determining the residual ferrous iron concentration at various intervals. The 1-10 phenathroline method of Vogel (1989) was used. In order to determine ferrous iron concentration a 10 µl sample was placed in tube and diluted with 1 ml of distilled water. The pH was adjusted to between 3.0 and 6.0 with 2 molar sodium acetate. 0.8 ml of 1-10 phenathroline solution was added and volume was made up to 10ml by adding distilled water. The absorbance at 515 nm was measured after 5-10 minutes. A calibration curve of known ferrous sulphate concentration was used to calculate the iron concentration. The concentration of iron (III) in solution was calculated by subtracting the average iron (II) concentration from total iron concentration measured at each point at the interval of one hour each.

P) Growth Profile of *Pseudomonas fluorescens*:
From overnight grown culture on nutrient agar plates single colony of Pseudomonas isolates were inoculated in freshly prepared nutrient broth and temperature was set as 32 ± 2 ^{0}C. Pseudomonas isolates inoculated nutrient broth tubes were agitated at 140 rpm and growth was monitored at regular interval by measuring the optical density at 660 nm in spectrophotometer.

Q) Growth of *Thiobacillus ferrooxidans* and *Pseudomonas fluorescens* in Presence of Metal Ions:
In order to test the resistance against metal ions ***Pseudomonas fluorescens*** and *Thiobacillus ferrooxidans* strains were incubated at 30 ±2 ^{0}C and 32 ±2 ^{0}C respectively with agitation speed of 140 rpm in the respective media using silicon, aluminum, titanium and iron. The metal ion resistant strains were selected by observing the growth in terms of turbidity at 660 nm and compared with un-inoculated control.

D) BIOEXTRACTION OF METALS FROM BAUXITE ORE:

1] Metal Extraction by Adapted and Non-Adapted Strains *Thiobacillus ferrooxidans*
Two kinds of strains were used in this study. One was the original type cultured in 9K medium containing ferrous ions. The other one was adapted, to higher dissolved metal concentration (Al and Fe) by repeated sub-culturing in 9K medium in the presence of different concentrations of added metal salts in the medium to grow in the medium containing bauxite ore (Kai et al., 1995). It could grow at a concentration of 2 g/ 100 ml of bauxite ore. The method of Shahverdi et al. (2001) was used for adaptation to bauxite ore. Bioleaching experiment was carried out by shake flask method in 250 ml Erlenmeyer flasks containing 9K medium. It was autoclaved at 121 ^{0}C for 15 minutes and pH was adjusted to 2.0 with 10 N H_2SO_4. The flasks were inoculated with 60 % of adapted *Thiobacillus ferrooxidans* inoculum (2 x 10^4/ml maximum cell biomass concentration). 60 % of non-adapted *Thiobacillus ferrooxidans* (2 x 10^4/ml) was inoculated as control. Sterile distilled water was added instead of inoculum in another flask which was kept as blank. All the flasks were incubated at 34 ± 2 ^{0}C at room temperature on a rotary shaker at 140

rpm. Experiment was run for 21 days. At the end of experiment pH was measured. The contents of the flasks were filtered and biomass of the bacterial cells and bauxite ore remained on the filter paper was weighed. At the termination of experiment analysis of bioextracted metals was carried out by its respective spectrophotometric method developed by Raquel et al. (2008) for aluminum and Demirhan and Fikriye (2007) for iron.

Aluminum was analyzed by taking 20 µl leached samples from the filtrate was added with 20 µl of H_2SO_4. The volume was made upto 3ml with doubled distilled water. 500 µl of 15 % sodium acetate was added to it for adjusting reaction pH. 200 µl of 0.1 % ascorbic acid was added to overcome the interference of iron. Lastly 200 µl of chrome azurol-s (0.04 % working solution diluted from stock solution of 0.1 %) was added to it. The whole assembly was incubated for 10 min. for violate colour development the absorbance was measured at 545 nm wavelength. For standard curve aluminum potassium sulphate was used.

The iron was detected from the filtrate by taking 20 µl leached sample and digested with 20 µl 10N H_2SO_4. The volume was made upto 3 ml with doubled distilled water. 1ml of Hydroxylamine hydrochloride (10 %) and 8ml of sodium acetate (10%) and 10 ml of 1, 10 phenanthroline solution (0.1 %) were added to it. The whole mixture was diluted to 100 ml with distilled water and allowed to stand for 10 min. for developing brick red colour. The absorbance was measured at 510 nm wavelength. The standard curve was prepared by taking ferrous ammonium sulphate.

2] Metal Extraction by Adapted and Non-Adapted Strains of *Pseudomonas fluorescens*

In order to extract metals from bauxite ore the bacterium, ***Pseudomonas fluorescens*** was adapted to bauxite ore according to method of Brandl and Faramarzi (2006). 150 ml of Kings medium containing 2g of bauxite ore was transferred to the Erlenmeyer flask, pH of the sample was adjusted to 7.2. The acclimatization of bacteria to bauxite ore was done at ambient temperature under continuous agitation. This adapted one ml culture of ***Pseudomonas fluorescens*** was transferred to newly make Kings medium and incubated for 10 days.

Bioleaching experiment was carried out by shake flask method in Erlenmeyer flask containing sterilized Kings medium. The flasks were inoculated with the 60 ml of adapted ***Pseudomonas fluorescens*** as well as other two systems were inoculated with 60 ml of non-adapted ***Pseudomonas fluorescens*** while in another one sterile distilled water. The volume was made 100ml by adding 40 ml Kings medium in each flask. The flasks were incubated at 32 ±2 ^{0}C on a rotary shaker at 140 rpm for one month. At the end of experiment pH was measured. The analysis of bioextracted metals was carried out by respective spectrophotometric method as earlier.

F) GENETIC IMPROVEMENT OF METAL EXTRACTING MICROBES:

1] Genetic Improvement of *Thiobacillus ferrooxidans* by Mutagenesis:

A) Mutagenesis through Ultra-Violet (UV) Irradiation:

Efficient *Thiobacillus ferrooxidans* mutants were developed as follows. Isolated *Thiobacillus ferrooxidans* was cultured in 250 ml Erlenmeyer flask containing 24 hours grown 9K culture medium and the same was used for various UV exposures. Ultra-violet mutagenesis was carried out according to Ling et al. (2007). The UV-light was switched on and allowed to pre-expose for ten minutes. 10 ml of pre-cultured suspension of the adapted *Thiobacillus ferrooxidans* was transferred in the Petri dishes under sterile condition. A distance of 60 cm was kept between the plates and UV-light source. *Thiobacillus ferrooxidans* was UV-irradiated for period of 10 and 20 minutes at a power of 30 W. During UV-exposure the suspension was constantly shaken with the help of mechanical vibrator. After UV-irradiation the aliquots were transferred to dark room for avoiding photo-reactivation. The overnight grown culture was diluted and spreaded on the newly made 9K solid medium (Silverman and Lundgren, 1959). The culture without UV-

Irradiation was used as control. After 10 days, percent frequency and survival percentage of mutants were calculated by comparing with control. The randomly grown colonies were selected on the basis of colony size, shape and colour. Selected mutant colonies were streaked on the slants and preserved in refrigerator at 4 ^{0}C until use. The same were continued for further use.

B) Mutagenesis through Ethyl Methane Sulphonate (EMS):
In order to make the efficient strains of *Thiobacillus ferrooxidans* genetic improvement was carried out by chemical mutagen EMS. Genetic improvement programme was carried out according to the method employed by Gohel et al. (2004).

10 ml of 24 hour grown cell suspension (10^6 cells/ml) of the *Thiobacillus ferrooxidans* was subjected to final concentration of EMS at 5 and 10 µg /ml for 60 minutes. To avoid toxic effects of the mutagen, cells were washed several times with sterile distilled water and were centrifuged at 4000 rpm for 10 minutes and decanted. Washed EMS treated cells were finally suspended in newly made 9K broth medium. Well mixed suspensions were diluted serially and spreaded on 9K solid medium. The plates were incubated at 36 ^{0}C for 10 days. The culture without treatment (mother strain) was used as control. The plates were incubated at 36 ^{0}C and colony counts were taken after 10 days of incubation. Percent survival and percent frequency was estimated by comparing with control colonies. The well established 30 colonies were selected randomly and cultured in 9K broth.

After 48 hours of incubation at 36 ^{0}C, the broth was used as inoculum for bioleaching experiments. To select the efficient mutants, bioleaching experiment was carried out as earlier and efficient mutants were selected and cultivated for multiplication and used further.

2] Genetic Improvement of *Pseudomonas fluorescens* by Mutagenesis:

A) Mutagenesis through UV-Irradiation:
Pseudomonas fluorescens cultured in 250 ml Erlenmeyer flask containing 24 hours grown culture in King's medium. Ultra-violet (UV) mutagenesis was carried out according to Saha and Bhattacharyya (1999). The UV-light was switched on and allowed to pre-expose for ten minutes. 10 ml of pre-cultured suspension of the adapted ***Pseudomonas fluorescens*** was transferred in the Petri dishes under sterile condition. A distance of 60 cm was kept between the plates and UV-light source. ***Pseudomonas fluorescens*** was UV-irradiated for period of 10 and 20 minutes at a power of 30 W. During UV-exposure the suspension was constantly shaken with the help of mechanical vibrator. After UV-irradiation the aliquots were transferred to dark room to avoid photo-reactivation for overnight by wrapping the plates in an aluminum foil. Overnight grown culture was diluted and spreaded on the newly made solidified Kings' medium (Kings, 1954). The culture without UV-Irradiation was used as control. After 10 days percent frequency and survival percentage was calculated by comparing with control. The 24 UV-mutants were selected on the basis of colony size, shape and colour and streaked on the slants and incubated at 32 ± 2 ^{0}C. The well grown slants were preserved in refrigerator and these were used further for the selection of better mutant.

B) Mutagenesis through Ethyl Methane Sulphonate (EMS):
In order to make efficient strains for metal bioleaching genetic improvement was carried out by chemical mutagenesis with EMS according to the method employed by Miller and Kokjohn (1990).

10 ml of 24 hours grown cell suspension (10^6 cells/ml) of the ***Pseudomonas fluorescens*** was subjected to final concentration of chemical mutagen EMS as 5 and 10 µg /ml for 60 minutes to cause maximum killing. To avoid the toxic effects of EMS, cells were washed several times with sterile distilled water and centrifuged at 4000 rpm for 10 minutes and decanted. Washed EMS treated cells were finally suspended in newly made Kings broth medium and well mixed

suspension was diluted serially and plated on Kings solid medium. The inoculated plates were incubated at 32 ^{0}C for 48 hours. The non treated mother strain was used as control. The plates were incubated at 32 ^{0}C and colony counts were taken after 48 hours of incubation. Percent survival and percent frequency was calculated by comparing with control colonies. Well established 24 colonies were selected randomly and used for bioleaching experiment further for the selection of better mutant.

G) EXTRACTION OF METALS BY GENETICALLY IMPROVED MICROBES FROM BAUXITE

1] Extraction of Metals by *Thiobacillus ferrooxidans* Mutants:

A) Extraction of Metals by *Thiobacillus ferrooxidans* UV Mutants:

Bioleaching experiment was carried out according to method employed by Ling et al. (2007). Each UV-mutant was cultured in separate flask for bioleaching experiment.

Thiobacillus ferrooxidans UV-mutants were cultivated in 9K medium (Silverman and Lundgren, 1959). The pH of the medium was adjusted to 2.0 with 10N H_2SO_4. The medium was inoculated with 24 hours old culture of *Thiobacillus ferrooxidans* UV-mutants. The culture was grown under sterile condition in 250 ml Erlenmeyer flasks on a rotary shaker (140 rpm) and incubated at 36 ^{0}C ± 2 ^{0}C. Pre weighed 2g bauxite ore was added in Erlenmeyer flask. In control flask, mother strain (without UV-Irradiation) was used as an inoculum with 60 ml of 9K medium along with 2g of bauxite ore and incubated like mutants. The aerobic condition was maintained by applying non absorbent cotton to the mouth of the flasks.

Experiment was run for one month. At the end of the experiment contents of each Erlenmeyer flask were filtered through whatman no.41 filter paper. Analysis of aluminum and iron was carried out according to spectrophotometric methods (Raquel et al., 2008; Demirhan and Fikriye 2007).

B) Extraction of Metals by *Thiobacillus ferrooxidans* EMS Mutants:

The bioleaching experiment was carried out in 9K medium. The pH of the medium was adjusted to 2.0 with 10N H_2SO_4. The medium was inoculated with 24 hours old culture of *Thiobacillus ferrooxidans* EMS-mutants. Pre weighed 2g bauxite ore was added in Erlenmeyer flask while in control flask inoculum was replaced by a 40 ml 9K medium along with 60 ml of sterile distilled water. The aerobic condition was maintained by applying non absorbent cotton to the mouth of flask. The cultures were grown under sterile condition in 250 ml Erlenmeyer flasks on a rotary shaker (140 rpm) and incubated at 36 ± 2 ^{0}C. The same condition was maintained for both the systems.

The experiment was run for one month. At the end of the experiment the contents of each Erlenmeyer flask were filtered through whatman no.41 filter paper. Aluminum and iron extraction in each case was estimated at the termination of experiment by spectrophotometric methods developed by Demirhan and Fikriye (2007) and Raquel et al. (2008) respectively.

After comparing both the above mutants the mutant UV- 26 was selected for further studies.

2] Extraction of Metals by *Pseudomonas fluorescens* Mutants:

A) Extraction of Metals by *Pseudomonas fluorescens* UV Mutants:

Bioleaching experiment was carried out according to the method employed by Ling et al. (2007). Each UV-mutant was cultured in separate flask for bioleaching experiment.

The effectiveness of UV-mutants in the bioextraction was studied in the shake flask method. The experiment was carried out in Erlenmeyer flasks containing 40 ml of Kings medium (Kings, 1954) [composition g/l protease peptone-20, Glycerin -15ml, Magnesium sulphate $MgSO_47H_2O$ -5.0, Di-Potassium hydrogen phosphate K_2HPO_4 - 2.5, Agar agar – 20, Distilled water – 1000ml pH – 7.2]. 60 ml inoculum from 24hr old culture (v/v) and 2 % bauxite ore

(loading mesh size of 0.20- 0.40 mm) was used. The aerobic condition of the system was maintained by putting non-absorbent cotton to the mouth of the flask. In control, same condition was maintained. Instead of UV-mutant mother strain (without treatment) was used as inoculum. The flasks were incubated under shaking conditions at 140 rpm at 32 ^{0}C ± 2 ^{0}C temperature. Experiment was carried out for 21 days. At the termination of experiment, assessment of bioleaching of aluminum and iron in the solution was done by spectrophotometric method of Raquel et al. (2008) and Demirhan and Fikriye (2007) respectively.

b) Extraction of metals by *Pseudomonas fluorescens* EMS mutants:

The effectiveness of EMS-mutants on the extraction was studied in the shake flask method. Experiment was carried out in Erlenmeyer flask containing 40 ml of Kings medium (Kings, 1954), 60 ml of inoculum (v/v) and 2 % bauxite ore concentration (loading mesh size of 0.20-0.40 mm). Inoculum was taken from 24 hours old culture. The aerobic condition of the system was maintained by putting non-absorbent cotton to the mouth of the flask. The flasks were incubated under shaking condition at 140 rpm at 32 ± 2 ^{0}C temperature. Experiment was carried out for 21 days. At the termination of experiment bioleaching of aluminum and iron was assessed as earlier. By comparing the performance of ***Pseudomonas fluorescens*** mutants UV-24 was selected as better and used for further studies.

H) EFFECTS OF ENVIRONMENTAL FACTORS ON BIOEXTRACTION OF METALS FROM BAUXITE ORE:

1] Effect of Media Composition on Metal Extraction by *Thiobacillus ferrooxidans*:

In order to assess the magnitude of media composition on bioleaching of metals from bauxite ore the present experiment was carried out. Five different media were used to optimize the effectiveness of media for bioleaching process. To study the effect of media composition, shake flask method was used.

A) Mineral Salt Medium

The experiment was carried out in 250 ml Erlenmeyer flasks containing 40ml Mineral salt medium [composition g/l - Ammonium sulphate [$(NH_4)_2SO_4$] -0.20, Magnesium sulphate [$MgSO_4.7H_2O$] -0.40, Di Potassium hydrogen phosphate [K_2HPO_4]-0.10, Potassium chloride [KCl] -0.10, Ferrous sulphate [$FeSO_4$] -22 , distilled water- 1000 ml, pH was adjusted to 4.5 with 1N H_2SO_4.] (Marsh and Norris, 1983).

B) Basal Salt Medium

Ammonium sulphate [$(NH_4)_2SO_4$] -800, Magnesium sulphate [$MgSO_4.7H_2O$] -160, Di-potassium hydrogen phosphate [K_2HPO_4]-400, Manganese chloride [$MnCl_2.4H_2O$] -0.10, Zinc chloride [$ZnCl_2$]- 70, Cobalt chloride [$CoCl_2.6H_2O$] -0.12, Boric acid [H_2BO_3]- 31, Sodium molybdate [$Na_2MoO_4.2H_2O$] -85.2 pH-6.5]

C) T and K Medium

Solution A – 800 ml and Solution B- Ferrous sulphate solution- 200 ml

Solution A-800ml

[Ammonium sulphate [$(NH_4)_2SO_4$]-0.4 g, Magnesium sulphate [$MgSO_4.7H_2O$]-0.4 g, Di-potassium hydrogen phosphate [K_2HPO_4]-0.4 g, Distilled water -800ml, pH 1.8 was adjusted with 1 N H_2SO_4]

Solution B-200ml [Ferrous sulphate ($FeSO_4$)-33.3 g and Distilled water- 200 ml]

Before adding the salt in the ferrous sulphate solution pH was adjusted with 1 N sulphuric acid at 1.8. Solutions A and B were autoclaved separately at 121^{0}C for 20 min. solution A and B were mixed to prepare T and K medium in aseptic condition.

d) Modified 9K medium (Glucose)

Solution -A (Basal salt solution- 600 ml)

Ammonium sulphate [$(NH_4)_2SO_4$]-2.0 g , Magnesium sulphate [$MgSO_4.7H_2O$]-0.5 g, Di-potassium hydrogen phosphate [K_2HPO_4]-0.05 g, Potassium chloride [KCl] – 0.1 g, Calcium nitrate – [$Ca(NO_3)_2$] – 0.01 g, Sulphuric acid [1 N H_2SO_4] and Distilled water -600ml.

Solution B - 300 ml (ferrous sulphate solution)

[Ferrous sulphate ($FeSO_4$)-33.3 g and Distilled water- 200 ml]

The pH of distilled water was adjusted to 2.5 followed by dissolution of ferrous sulphate and filtered. Filtrate was sterilized at 121^0C for 10 minutes.

Solution -C (Glucose solution)

Glucose 10 gm, Distilled water- 100 ml followed by filtration and sterilization at 121^0C for 5 minutes. For preparing liquid 9K Glucose medium, solution A, B and C were mixed aseptically.

e) 9K medium

(Silverman and Lundgren, 1959) [composition g/l Ammonium sulphate $(NH_4)_2SO_4$ -3.0, Magnesium sulphate $MgSO_47H_2O$ -0.5, Di-potassium hydrogen phosphate K_2HPO_4 - 0.5, Potassium chloride KCl- 0.1, Calcium nitrate $Ca(NO_3)_2$ -0.01, Ferrous Sulphate $FeSO_4.7H_2O$-21] autoclaved at 121 ^{0}C for 15 minutes and pH of the medium was adjusted to 2.0 with 10 N H_2SO_4 .

2] Effect of PH on Metal Extraction:

A) By *Thiobacillus ferrooxidans*:

To study the effect of pH on bioextraction of metals from bauxite ore the experiment was carried out by shake flask method employed by Chen and Lin (2001) *Thiobacillus ferrooxidans* was cultivated in 9K medium (Silverman and Lundgren, 1959). The medium was autoclaved at 121 ^{0}C for 15 minutes to prevent interference of other microorganisms.

To optimize the appropriate pH the pH of the medium were adjusted to1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0, with 10N H_2SO_4. The medium was inoculated with approximately 5 x 10^6 cells / ml (maximum cell biomass concentration). The culture was grown under sterile condition in 250 ml Erlenmeyer flasks on a rotary shaker (140 rpm) and incubated at 36 ^{0}C ± 2 ^{0}C preweighed 2g bauxite ore was added in each Erlenmeyer flask. In control flask, inoculum was replaced by 40 ml of 9K medium along with 60 ml of sterile distilled water. The aerobic condition was maintained by applying non absorbent cotton to the mouth of the flasks. The same condition was maintained for control system.

The experiment was run for one month. At the end of the experiment the final pH was measured. The contents of each Erlenmeyer flask were filtered through whatman no.41 filter paper. Aluminum and iron extracted were measured as earlier.

B) By *Pseudomonas fluorescens*:

To study the effect of pH on bioextraction of metals shake flask method was employed. Microbial solublization of metals from bauxite ore was evaluated using 250 ml Erlenmeyer flasks incubated with a 24 hours old culture of adapted **Pseudomonas fluorescens**. The flasks containing 40 ml Kings medium with varying initial pH such as 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10, 11, and 12 were taken for the experiment. The medium was autoclaved at 121 ^{0}C for 15 minute and the initial pH was maintained constant throughout the incubation period. The flasks containing 60 ml of ***Pseudomonas fluorescens*** inoculum (maximum biomass concentration of 1.4 x 10^6 cells/ml) and 2 % of initial concentration of bauxite ore were incubated at constant 32 ± 2 ^{0}C temperature along with continuous shaking at 140 rpm. The aerobic condition was maintained by putting non absorbent cotton to the mouth of flasks (Hossain et al., 2004). The measurement of extracted aluminum and iron was done as earlier.

3] Effect of Temperature on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:

Bioleaching experiment was carried out in 250 ml Erlenmeyer flasks containing 40 ml 9K (Silverman and Lundgren, 1959) medium. The medium was autoclaved at 121 ^{0}C for 15 minutes and pH of the medium was adjusted to 2.0 with 10N H_2SO_4. 60 ml of inoculum (2.0 x 10^4 cells) was added. The pulp density of bauxite ore was 2 %. The flasks were incubated at different temperatures such as 4 ^{0}C, 10 ^{0}C, 20 ^{0}C, 24 ^{0}C, 28^{0}C, 32^{0}C, 36^{0}C, 40^{0}C, 44 ^{0}C and 48^{0}C with constant shaking speed at 140 rpm. The experiment was run for one month. At the end of the experiment final pH was measured and contents of the flasks were filtered. Analysis of metals was carried out by spectrophotometric methods as earlier.

B) By *Pseudomonas fluorescens*:

In order to study the effect of temperature on extraction of metals from bauxite ore bioleaching experiment was carried out in 250 ml Erlenmeyer flasks containing the 40 ml Kings medium. The medium was autoclaved at 121 ^{0}C for 15 minutes and 60 ml of inoculum (2.0 x 10^4 cells) and 2 g bauxite ore was added to the flasks. The flasks were incubated at different temperatures such as 10 ^{0}C, 15 ^{0}C, 20 ^{0}C, 24 ^{0}C, 28^{0}C, 32^{0}C, 36^{0}C, 40^{0}C, 44 ^{0}C and 48^{0}C with constant shaking speed at 140 rpm. The experiment was run for 21 days and at the end of experiment, the final pH was measured and contents of the same were filtered. Analysis of aluminum and iron content was carried out as earlier.

4] Effect of Residence Time on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:

Bioleaching experiment was carried out in 250 ml conical flasks containing 40 ml of 9K (Silverman and Lundgren, 1959) medium. The medium was autoclaved at 121 ^{0}C for 15 minutes and initial pH 2.0 was adjusted with H_2SO_4. 60 % of the inoculum (maximum cell biomass concentration of 2 x 10^7 cells/ml) and 2 gm of bauxite ore (0.20 to 0.40 mm particle size) was added to it. The aerobic condition of the system was maintained by putting non absorbent cotton to the mouth of the flasks. The flasks were agitated on an orbital shaker at 140 rpm at 34 ± 2 ^{0}C. In the control same condition was maintained but instead of inoculum 60 ml distilled water was added. Other conditions were similar to those applied for the maintenance of cultures. Flasks were taken out on a regular basis that is after 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 days. Analysis of bioleached metals aluminum and iron was done by spectrophotometric methods described earlier.

B) By *Pseudomonas fluorescens*:

The effect of residence time on the bioextraction of metals was carried out by shake flask method. Bioleaching experiment was carried out in 250 ml conical flasks containing 40 ml of Kings medium. After autoclaving the medium 60 ml of inoculum (maximum cell biomass concentration of 2 x 10^7 cells/ml) and 2 g of bauxite ore (of 0.20 to 0.40 mm particle size) were added to it. The flasks were agitated on shaker at 140 rpm at 32 ± 2 ^{0}C. The flasks were taken out on a regular basis i.e. after 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 days and bioleached aluminum and iron were analyzed as earlier.

5] Effect of Substrate Concentration on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:

To assess the effect of substrate concentration on bioleaching of metals the experiment was carried out by shake flask method employed by Chen and Lin, (2001). *Thiobacillus ferrooxidans* was cultivated in 9K medium (Silverman and Lundgren, 1959) and pH was adjusted to 2.0 with 10N H_2SO_4 and the medium was inoculated with approximately 5 x 10^6 cells / ml maximum cell biomass concentration. The culture was grown under sterile condition in 250 ml Erlenmeyer flasks on a rotary shaker (140 rpm) and incubated at 36 ^{0}C ± 2 ^{0}C.

After incubation of four days Pre weighed bauxite ore was added in Erlenmeyer flasks such as1.0, 2.0, 4.0, 5.0, 6.0, 8.0, 9.0 and 10 gm/100 ml respectively. In control flask, inoculum was replaced by a 40 ml of 9K medium along with 60 ml of sterile distilled water and same condition was maintained for control system. The experiment was run for one month. At the end of the experiment final pH was measured. The contents of each Erlenmeyer flasks were filtered through whatman no.41 filter paper. The aluminum and iron extraction in each case was measured as earlier.

B) By *Pseudomonas fluorescens*:

To study the effect of substrate concentration on bioleaching of metals, the experiment was carried out by shake flask method employed by Chen and Lin (2001). The medium was inoculated approximately 5 x 10^6 cells / ml (maximum cell biomass concentration). The culture was grown under sterile condition in 250 ml Erlenmeyer flasks on a rotary shaker (140 rpm) and incubated at 32 ± 2 ^{0}C. After incubation of 24 hours, pre weighed bauxite ore was added in Erlenmeyer flask such as 2.0, 4.0, 6.0, 8.0, 10, and 12 g/100 ml. In control flask the inoculum was replaced by a 40 ml of Kings Medium along with 60 ml of sterile distilled water and same condition was maintained. Experiment was run for 21 days. At the end of each experiment the final pH was measured. The content of each Erlenmeyer flask was filtered through Whatman no.41 filter paper. The extraction of aluminum and iron measured as per the methods described earlier.

6] Effect of Agitation on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:

The effect of agitation on the bioextraction of metals from bauxite ore was studied in the shake flask method. The experiment was carried out in Erlenmeyer flasks containing 40 ml of 9K (Silverman Lundgren, 1959) medium. Inoculum 40 % (v/v) taken from 4 days old culture (maximum biomass concentration of 2.0 x 10^6 /ml and 2 % bauxite ore concentration was added to it. The pH of the media was adjusted with 10N H_2SO_4. The aerobic condition of the system was maintained by putting non-absorbent cotton to the mouth of the flasks. The flasks were incubated at various shaking conditions such as a 60, 80, 120 and 140 rpm at 36 ^{0}C ± 2 ^{0}C temperature. The experiment was carried out for one month. At the termination of experiment aluminum and iron were estimated by spectrophotometry described as earlier.

B) By *Pseudomonas fluorescens:*

The effect of agitation on the bioextraction of metals was studied by the shake flask method. Experiment was carried out in Erlenmeyer flasks containing 40 ml of Kings medium 60 ml (v/v) inoculum (maximum cell biomass concentration of 2.0 x 10^6 /ml) and 2 % bauxite ore (loading mesh size of 0.20- 0.40 mm). Inoculum was taken from 24 hour old culture. The flasks were incubated at various shaking conditions such as a 60, 80, 120 and 140 rpm speed. The control was kept stationary at 32 ± 2 ^{0}C temperature. Experiment was carried out for 21 days. The extracted aluminum and iron were estimated by spectrophotometry as per previous steps.

7] Effect of Particle Size of the Substrate (Mesh Size) on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:

In order to study the effect of bauxite ore size on bioextraction of metals shake flask method was employed (Hossain et al., 2004). The collected bauxite ore was ground and crushed to fine particles and screened to select the different sizes such as 0.20-0.40, 0.80-120, 120-2.00, 280-4.00, 4.00-5.00 and 5.00-6.00 mm.

Shake flask experiment was carried out in 250 ml Erlenmeyer flasks containing 40 ml of 9K medium (Silverman and Lundgren, 1959) the medium was autoclaved at 121 ^{0}C for 15 minutes and pH was adjusted to 2.0 with 10 N H_2SO_4. Each flask received 60 % of maximum adapted *Thiobacillus ferrooxidans* (Biomass concentration of 1.4 x 10^4 cell/ml). Inoculum was added in

sterile condition. In control, 60 % sterile distilled water was added instead of inoculum. The flasks were kept for 30 days at 36 ± 2 ^{0}C temperature with continuous 140 rpm shaking condition. At the termination of experiment, pH was measured and sample was filtered through whatman no.41 filter paper. Solubilized filtrates were subjected to metal analysis by spectrophotometric methods as earlier.

B) By *Pseudomonas fluorescens*

The effect of bauxite ore size on bioextraction of metals was studied by shake flask method employed by Hossain et al. (2004). The Bauxite ore was ground and crushed to fine particles and screened to select the varying sizes such as 0.20-0.40, 0.80-120, 120-2.00, 280-4.00, 4.00-5.00 and 5.00-6.00 mm.

In each 250 ml Erlenmeyer flasks 60 ml of inoculum (24 hour old culture) of ***Pseudomonas fluorescens*** and 40 ml of Kings medium were mixed. To it 2g of bauxite ore of above particle sizes were added in the respective flasks. The flasks were incubated and at 32 ± 2 ^{0}C for 21 days with constant shaking at 140 rpm. At the termination of experiment aluminum and iron were estimated.

8] Effect of Inoculum Concentration on the Extraction of Metals:

A) *Thiobacillus ferrooxidans* Inoculum:

The experiment was carried out in 250 ml Erlenmeyer flasks containing 9K medium (Silverman and Lundgren, 1959). The medium was autoclaved at 121 ^{0}C for 15 minutes to prevent interference of indigenous microorganisms. The pH was adjusted to 2.0 with 10 N H_2SO_4. Each flask was inoculated with various concentrations of inoculii such as 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 % of eight days grown old culture of well adapted *Thiobacillus ferrooxidans* (Biomass concentration of 1.4 x 10^6 cell/ml). Inoculii were added in sterile condition. In control 60 % sterile distilled water was added instead of inoculum. The aerobic condition was maintained by putting non-absorbent cotton at the mouth of leaching flasks. The flasks were kept for 30 days at 36 ± 2 ^{0}C temperature with continuous 140 rpm shaking condition. At the termination of experiment pH was measured and sample was filtered through whatman no.41 filter paper. The extracted aluminum and iron were analyzed as earlier.

B) *Pseudomonas fluorescens* Inoculum:

In order to study the effect of bacterial inoculum concentration on extraction of metals shake flask method was employed (Hossain et al., 2004). The bauxite ore was ground and crushed to fine particles and 2% pulp concentration was added to the 250 ml Erlenmeyer flasks. Each flask was inoculated with various concentrations of inoculii such as10 , 20, 30, 40, 50, 60, 70, 80, 90 and 100 % of eight days grown old culture of ***Pseudomonas fluorescens*** (biomass concentration of 1.4 x 10^6 cell/ml) along with control. In control 60 ml sterile distilled water was added instead of inoculum. Flasks were kept for 21 days at 32 ± 2 ^{0}C temperature with continuous 140 rpm shaking speed in aerobic condition. Estimation of aluminum and iron was done as above.

9] Effect of Sodium Chloride (NaCl) Concentration on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:

In order to study the salinity effect on bioextraction of metals from bauxite ore shake flask method was employed. Microbial solubilization of metals from bauxite ore was evaluated using 250 ml Erlenmeyer flask containing 40 % of 9K medium (Silverman and Lundgren, 1959) the medium was autoclaved at 121 ^{0}C for 15 minutes with varying concentration of sodium chloride (NaCl) such as 0.5 %, 1.0 %, 1.5 %, 2.0 %, 2.5 %, 3.0 %, 3.5 %, 4.0 %, 4.5 % and 5.0 %. Initial pH of the medium was adjusted to 2.0 with 10 N H_2SO_4. Flasks were also contain 60 % of successively adapted inoculum of *Thiobacillus ferrooxidans* with maximum biomass concentration of 1.4 x 10^6 cells/ml and 2 % of initial loading concentration of bauxite ore.

The flasks were incubated at 36 ±2 ^{0}C constant temperature along with 140 rpm continuous shaking condition for one month. The aerobic condition was maintained by putting non absorbent cotton at the mouth of flasks (Hossain et. al., 2004). At the end of each experiment pH was measured and contents were filtered using whatman filter paper no.41. Filtrates were analyzed for aluminum and iron extraction by spectrophotometric methods described as earlier.

B) By *Pseudomonas fluorescens:*

To study the salinity effect on bioextraction of metals from bauxite ore shake flask method was employed. Microbial solublization of metals from bauxite ore was evaluated using 250 ml Erlenmeyer flask containing 40 ml of Kings medium. The medium was autoclaved at 121 ^{0}C for 15 minutes with varying concentration of sodium chloride (NaCl) such as 0.5 %, 1.0 %, 1.5 %, 2.0 %, 2.5 %, 3.0 %, 3.5 %, 4.0 %, 4.5 % and 5.0 %. Flasks were also containing 60 % inoculum of ***Pseudomonas fluorescens*** with maximum biomass concentration of 1.4 x 10^6 cells/ml. 2 % of initial concentration of bauxite ore was added to it. The flasks were incubated at 32 ± 2^{0}C of constant temperature along with 140 rpm continuous shaking condition with residence time of 21 days. Aluminum and iron was estimated as earlier.

I) EFFECT OF CELL METABOLITES ON BIOEXTRACTION OF METALS FROM BAUXITE ORE:

A) Using *Thiobacillus ferrooxidans* Cell Free Extract:

For studying the role of metabolic products (cell extracts) in the solublization of metals (Kim et al., 2005) a shake flask method was employed. Leaching experiment with culture filtrate of *Thiobacillus ferrooxidans* grown in 9K (Silverman Lundgren, 1959) medium for 21 days at 36 ^{0}C ± 2 for constant shaking condition of 140 rpm and was centrifuged at 10,000 g for 10 minutes and collected supernatant was used for metal extraction. The same condition was maintained for the leaching flask which received *Thiobacillus ferrooxidans* cell suspension. The bioleaching experiments with culture filtrate was carried out in 250 ml Erlenmeyer flasks with continuous shaking at 140 rpm. The bauxite ore concentration was 2 % w/v (loading mesh size of 0.20- 0.40 mm). The pH of the medium was adjusted to 2.0 with 10N H_2SO_4. A conical flasks with 9K medium autoclaved at 121 ^{0}C for 15 minute was set as a control which contain bauxite ore 2 % and pH was adjusted to 2.0 with 10 N H_2SO_4. Distilled water was added instead of cell extract (culture filtrate) in set control flask. The all the flask was incubated at 34 ± 2 ^{0}C temperature. The experiment was run for 21 days. At the end of experiment contents were filtered as usual and filtrate was analyzed by spectrophotometry described as earlier.

B) Using *Pseudomonas fluorescens* Cell Free Extract:

In order to study the role of metabolic products (cell extracts) in the solublization of metals, the experiment was conducted according to method of Kim et al. (2005). Leaching experiment with culture filtrate of ***Pseudomonas fluorescens*** was carried out in 250 ml conical flasks containing 100 ml of cell free extract (10 days grown culture of ***Pseudomonas fluorescens*** was used for preparing cell free extract by centrifugation at 10,000 rpm for 10 min. followed by filtration of supernatant through cintered glass filter). The bioleaching experiment with cell free extract was carried out with 2g bauxite ore concentration at 32 ± 2 ^{0}C for 21 days. At the end of experiment extracted aluminum and iron were measured spectrophotometrically

J) SYNERGISTIC EFFECT OF MICROBES ON BIOEXTRACTION OF METALS FROM BAUXITE ORE:

1] Combined Effect of Metal Extracting Microbes with Other Microbes:

A) *Thiobacillus ferrooxidans* and Other Bacteria**:**

To observe the impact of extragenous microbes on bioextraction of metals along with *Thiobacillus ferrooxidans* bioleaching experiment was carried out by shake flask method in 250 ml Erlenmeyer flask having 40 ml of modified 9K glucose medium.

The 9K was medium loaded with 2 % concentration of bauxite ore (particle size of 0.20 to 0.40 mm) and the flask received 30 ml of 48 hours grown culture of *Thiobacillus ferrooxidans* with maximum cell biomass concentration of (1.2 x 10^2 cells/ml) in combination with extragenous bacteria such as Bacillus megaterium, Bacillus polymyxa, Bacillus cereus, Pseudomonas aeruginosa and Pseudomonas cissicola. Bioleaching flask received 40 ml of 9K Glucose medium with 30 ml of inoculum of *Thiobacillus ferrooxidans* and 30 ml of any one extragenous bacterium. Each flask received 30 ml of inoculum of *Thiobacillus ferrooxidans* and other bacterium. The abiotic as well as biotic controls were cultivated under same conditions. In abiotic control inoculum was replaced by 60 ml sterile distilled water. In biotic control only *Thiobacillus ferrooxidans* was inoculated with 2 gm bauxite ore. These were incubated at 30 ± 2 ^{0}C with continuous shaking speed of 140 rpm. The experiment was run for 22 days. At the termination of experiment pH was measured. The content of the same was filtered through whatman no.41 filter paper and filtrate was subjected to spectrophotometric estimation of solubilized aluminum and iron (Raquel et al., 2008; Demirhan and Fikriye, 2007).

B) *Pseudomonas fluorescens* and Other bacteria:

Microbial solublization of metals from bauxite ore was evaluated using shake flask method which was carried out in 250 ml Erlenmeyer flask inoculated with a 24 hours grown culture of ***Pseudomonas fluorescens***. In the experiment ***Pseudomonas fluorescens*** was used alone and in combination with microorganisms. Inoculum was prepared in Kings medium 24 hours before use. Inoculum of *Pseudomonas sp.* prepared in Kings broth. For Bacillus sp. modified Broomfield medium was used [composition g/l Ammonium sulphate [$(NH_4)_2SO_4$] – 1.0, Magnesium sulphate [$MgSO_47H_2O$] -0.5, Potassium hydrogen phosphate [K_2HPO_4] - 0.5, Glucose – 20.00] (Broomfield, 1954).

As usual 6 bioleaching flasks received 60 ml Glucose mineral medium was taken in each and 30 ml of ***Pseudomonas fluorescens*** which was constant in all leaching flasks and in combination with one bacterium in each flask such as *Bacillus megaterium*, *Bacillus polymyxa, Bacillus cereus*, *Pseudomonas aeruginosa* and *Pseudomonas cissicola* was added to it. A control was set in which inoculum was replaced by 60 ml of sterile distilled water to assess the metal solublization.

The pH of the medium was adjusted to 6.5 with 10 N H_2SO_4 in addition a control in which ***Pseudomonas fluorescens*** alone was used as a bioleaching agent to compare with other bacteria. The bioleaching experiment was carried out 30 ± 2 ^{0}C with constant shaking condition of 140 rpm for one month. At the termination of experiment pH was measured and leached solutes with bacterial biomass were filtered with Whatman filter paper no. 41. Filtrate was analyzed by spectrophotometric detection methods for aluminum and iron (Raquel et al., 2008; Demirhan and Fikriye 2007).

C) *Thiobacillus ferrooxidans* and Fungi**:**

The present experiment was carried out to assess the impact of indigenous fungi on bioextraction of metals in association with the bacterium, *Thiobacillus ferrooxidans*. Bioleaching experiment was carried out by shake flask method in 250 ml Erlenmeyer flask having 40 ml of modified 9K glucose medium as above. The 9K Glucose medium was used to favour the growth of fungal isolates. The medium was autoclaved at 121^{0}C for15 minutes to prevent the interference of indigenous microorganisms.

The 9K medium was loaded with 2 % concentration of bauxite ore. The flask received 30 ml of 48 hours grown culture of *Thiobacillus ferrooxidans* with maximum cell biomass concentration of (2.0 x 10^6 cells/ml) in combination with indigenous fungal isolates. Indigenous fungal isolates of *Aspergillus niger, Aspergillus flavus, Penicillium sp., Fusarium sp. and Rhizopus sp.* were used along with *Thiobacillus ferrooxidans*. Fungal isolates were cultivated in medium

proposed by Rashid et al. (2001) [composition g/l: - Glucose- 22.00, $NaNO_3$-1.5, KH_2PO_4 -0.5, $MgSO_4.7H_2O$-0.025, KCl- 0.025 and yeast extract -0.1, Distilled water -1000 ml]. Glucose and mineral salt solution was sterilized separately at 121 ^{0}C for 15 min and 5 min. respectively. The pH of the medium was adjusted to 5.5 with 10N HCl to use as culture medium for the multiplication of fungal isolates each. Each flask received 30 ml inoculum of *Thiobacillus ferrooxidans* and 30 ml inoculum of fungal isolate. The abiotic as well as biotic controls were cultivated under same conditions. In abiotic control inoculum was replaced by 60 ml sterile distilled water. In biotic control only *Thiobacillus ferrooxidans* with 2 gm bauxite ore was added. The flasks were incubated at 30 ± 2 ^{0}C with continuous shaking speed of 140 rpm. The experiment was run for 22 days. At the termination of experiment pH was measured. The contents of the same were filtered through whatman no.41 filter paper and filtrate was subjected to spectrophotometric estimation of aluminum and iron by the method described earlier.

D) *Pseudomonas fluorescens* and Fungi:

To study the impact of indigenous fungal isolates on extraction of metals by heterotrophic bacterium ***Pseudomonas fluorescens*** bioleaching experiment was carried out by shake flask method in 250 ml Erlenmeyer flask having 40 ml of Glucose Mineral Medium [composition g/l Potassium Di- hydrogen phosphate (KH_2PO_4)-1.0, Magnesium sulphate ($MgSO_4.7H_2O$)-0.2, Calcium carbonate ($CaCO_3$) - 1.0, Sodium chloride (NaCl)-0.2, Ferrous sulphate ($FeSO_4$) -0.05, Sodium molybdate ($NaMoO_4$) -0.005 pH – 7.0 filter sterilized. Glucose was added to give final concentration of 20 %]. The medium was autoclaved at 121^{0}C for 15 minutes for sterilization.

The Glucose mineral medium was loaded with 2 % concentration of bauxite ore (particle size of 0.20 to 0.40 mm) and the flask received 30 ml of 48 hours grown culture of ***Pseudomonas fluorescens*** with maximum cell biomass concentration (1.5 x 10^8 cells/ml) in combination with indigenous fungal isolates. Indigenous fungal isolates of *Aspergillus niger, Aspergillus flavus*, *Penicillium sp. Fusarium sp. and Rhizopus sp.* were used along with **Pseudomonas fluorescens**. Fungal isolates were cultivated in medium proposed by Rashid et al. (2001) [composition g/l: - Glucose- 22.00, $NaNO_3$-1.5, KH_2PO_4 -0.5, $MgSO_4.7H_2O$-0.025, KCl- 0.025, and yeast extract -0.1, Distilled water -1000 ml. Glucose and mineral salt solutions was sterilized separately at 121 ^{0}C for 5 and 15 minutes respectively. The pH of the medium was adjusted to 5.5 with 10N HCl]. 48 hours grown cultures of fungi were used as an inoculii.

Each bioleaching flask received 40 ml of Glucose mineral salt medium with 30 ml of ***Pseudomonas fluorescens*** inoculum and 30 ml of fungal inoculum along with 2g bauxite ore. In control only ***Pseudomonas fluorescens*** with 2 gm bauxite ore was added. These were incubated at 30 ± 2 ^{0}C with continuous shaking speed of 140 rpm. The experiment was run for 21 days and at the end of experiment pH was measured. The contents of the same were filtered through Whatman no.41 filter paper and filtrate was analyzed by spectrophotometric estimation of solubilized metal ions.

K) MICOBIOLOGICAL ANALYSIS OF FLY ASH

Microbial population such as bacteria fungi and actinomycetes were analyzed by standard method for soil microbial populations and were expressed in terms of colony forming units (Black et al., 1965). Microbiological characterization of fly ash was conducted by serial dilution method (Clark, 1965). For isolation of bioleaching microorganisms, method employed by Acharya et al. (2004) was used. The soil samples were (0.5 to 1.0 kg) collected from thermal power station Parli. The collected fly ash samples were immediately transferred in the ziplock bags and stored at 4 ^{0}C in the refrigerator until use.

From the collected fly ash samples, 1 gm of fly ash was ground to fine powder and added in 100 ml distilled water. The solution was stirred and mixed well to separate fungal spores from fly ash particles. For isolation and enumeration of microbial isolates 1gm of fly ash was serially

diluted and 1ml of respective dilution was used for pour plating using the following specific media. The medium Rose Bengal Agar was used for fungi isolation [Composition g/l Dextrose-10, Peptone-5.0, $MgSO_4.7H_2O$ – 1.0, $FeSO_4$ -0.5, Rose Bengal -0.03, Agar-agar-20, Distilled water-1000ml, pH- 6.5] (Martin, 1950). The medium Nutrient agar was used for bacterial isolation [composition g/l peptone- 5.0, beef extract-3.0, glucose-10.0 agar -16.0, distilled water- 1000ml, pH-7.5] Jensen's agar medium was used for the isolation of actinomycetes [Composition g/l dextrose-2.0, caesine-1.0, $MgSO_4.7H_2O$ – 0.2, $FeCl_3$-trace, KH_2PO_4-0.5, distilled water- 1000ml pH-6 to 6.5] (Jensen, 1930).

Media were sterilized by autoclaving 121 ^{0}C for 15 minutes. Inoculated plates were incubated for 36 hours for bacterial isolation, and for fungal as well as actinomycetes 6 and 8 days respectively at 30 ± 2 ^{0}C. The growth was reported in the form of colony forming unit (c.f.u.).

L] BIOEXRACTION OF METALS FROM FLY ASH BY GENETICALLY IMPROVED MICROBES

1) Extraction by Adapted and None Adapted Mutant Strains of *Thiobacillus ferrooxidans*:
In order to extract metals from fly ash the bacterium was adapted to fly ash according to method of Tyagi et al. (1994) 150 ml of 9K medium containing 4gm of fly ash was transferred to the 250 ml Erlenmeyer flask. The pH of sample was adjusted to 4.0 with 10N H_2SO_4 and 0.75 g $FeSO_4$ was added to medium. For acclimatization of bacteria to industrial waste, the bacteria were incubated to ambient temperature (24 ± 2 ^{0}C) under continuous agitation. After initial adaptation, pH was found decreased to 2.5. This adapted one ml culture was transferred to newly made 9K medium (Silverman and Lundgren, 1959). The sample was incubated for four weeks. Red to dark-brown Coloration or precipitate indicated the presence of oxidative bacteria. Two control were used one without addition of the $FeSO_4$ and another with addition of 0.5 % $FeSO_4$ (Tyagi et al., 1994).

Bioleaching experiment was carried out by shake flask method in 250 ml Erlenmeyer flask containing sterilized 9K medium. The flasks were inoculated with the 60 ml of adapted *Thiobacillus ferrooxidans* (approximately 2 x 10^4/ml maximum cell biomass concentration) as well as other two systems were also set one inoculated with 60 ml of non-adapted *Thiobacillus ferrooxidans* (approximately 2 x 10^6/ml) while in another, sterile distilled water was added instead of inoculum. The flasks were incubated at 34 ± 2 ^{0}C on a rotary shaker at 140 rpm. Experiment was run for one month. At the end of experiment pH was measured. At the termination of experiment analysis of bioextracted metals (aluminum, iron and manganese) was carried out by their respective spectrophotometric methods developed by Raquel et al. (2008) for aluminum and Demirhan and Fikriye (2007) for iron. The solubilized manganese in the filtrate was quantified by Perkin Elmer (3100) Atomic Absorption Spectrophotometer (Acharya et al., 2004).

2) Extraction by Adapted and Non-Adapted Mutant Strains of *Pseudomonas fluorescens*:
In order to extract metals from fly ash the bacterium, ***Pseudomonas fluorescens*** was adapted to fly ash according to method of Brandl and Faramarzi (2006). 150 ml of Kings medium containing 4gm of fly ash was transferred to the Erlenmeyer flask, pH of the sample was adjusted to 7.2. The acclimatization of bacteria to fly ash was done at ambient temperature under continuous agitation. This adapted one ml culture of ***Pseudomonas fluorescens*** was transferred to newly made Kings Medium and incubated for 10 days.

Bioleaching experiment was carried out by shake flask method in Erlenmeyer flask containing sterilized Kings medium. The flasks were inoculated with the 60 ml of adapted ***Pseudomonas fluorescens*** as well as other two systems were inoculated with 60 ml of non-adapted ***Pseudomonas fluorescens*** while in another one sterile distilled water. The volume was made 100ml by adding 40 ml Kings medium in each flask. The flasks were incubated at 32 ±2 ^{0}C on a

rotary shaker at 140 rpm for one month. At the end of experiment pH was measured. The analysis of bioextracted metals was carried out by respective spectrophotometric method as earlier.

M] EFFECT OF ENVIRONMENTAL FACTORS ON BIOEXTRACTION OF METALS FROM FLY ASH:

1] Effect of pH on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:

To emphasize the effect of pH, experiment was carried out in Erlenmeyer flask. *Thiobacillus ferrooxidans* was cultivated in sterilized 9K medium. The pHs of the medium were adjusted to1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0, with 10N H_2SO_4. Pre weighed, washed and dried 4g coal fly ash was added in each Erlenmeyer flask. In control flask inoculum was replaced by a 40 ml of 9K medium along with 60 ml of sterile distilled water (Broambaacher et al., 1998). The experiment was run for one month and at the end metals were analyzed as per the methods described earlier.

B) By *Pseudomonas fluorescens:*

To emphasize the effect of pH experiment was carried out in Erlenmeyer flask containing 40 ml Kings medium and the pH was adjusted to 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0. in separate flasks. The 60 ml inoculum along with 4g coal fly ash was added in each flask. The flasks were incubated at 32 ± 2 ^{0}C at 140 rpm shaking speed for 21 days. At the end of experiment the content of each Erlenmeyer flask was filtered through whatman no.41 filter paper. The metals from the filtrate, aluminum, iron, and manganese were estimated as earlier.

2] Effect of Temperature on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:

To study the effect of temperature on extraction of metals from fly ash bioleaching experiment was carried out in 250 ml Erlenmeyer flasks containing 40 ml sterilized 9K medium. 4g fly ash was added in each flask and flasks were incubated at different temperatures such as 10 ^{0}C, 20 ^{0}C, 25 ^{0}C, 30^{0}C, 34^{0}C, 38^{0}C, 42^{0}C, 44 ^{0}C 46^{0}C and 48^{0}C with constant shaking speed at 140 rpm for one month. The extracted metals were measured as earlier.

B) By *Pseudomonas fluorescens:*

In order to study the effect of temperature on extraction of metals from fly ash, bioleaching experiment was carried out in 250 ml Erlenmeyer flasks containing the 40 ml Kings medium in each. The medium was sterilized at 121 ^{0}C for 15 minutes and 60 ml of inoculum (2.0 x 10^6 cells /ml) was added to it. The pH of the medium was adjusted to 7.2 the fly ash pulp density of 4 % w/v was added in each. Flasks were incubated at different temperatures such as 10 ^{0}C, 20 ^{0}C, 25 ^{0}C, 30^{0}C, 34^{0}C, 38^{0}C, 42^{0}C, 44 ^{0}C 46^{0}C and 48^{0}C with constant shaking speed at 140 rpm using incubator shaker. Experiment was run for 21 days and at the end of experiment, the final pH was measured and contents of the same were filtered. Analysis of metals was carried out by spectrophotometric method employed as earlier.

3] Effect of Residence Time on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:

For studying the effect of residence time on the extraction of metals bioleaching experiment was carried out in 250 ml conical flasks containing 40 ml of sterilized 9K medium inoculated with 60 ml of inoculum and 4 gm of pre weighed fly ash. The flasks were incubated at 34 ± 2 and at 140 shaking speed. In control, same condition was maintained but instead of inoculum, 60 ml of distilled water was added. Flasks were taken out on a regular basis i.e. after 2, 6, 10, 14, 20, and 24 days. Analysis of metal extraction was carried out by Spectrophotometric method described earlier.

B) By *Pseudomonas fluorescens:*

Bioleaching experiment was carried out in 250 ml conical flasks containing 40 ml of sterilized Kings medium and 4 % of pre weighed fly ash was added to the bioleaching flasks. The flasks were agitated on an orbital shaker at 140 rpm at 32 ± 2 ^{0}C. The aerobic condition of the system was maintained by putting non absorbent cotton to the mouth of the flasks. In control same condition was maintained but instead of inoculum 60 ml distilled water was added. Flasks were taken out on a regular basis that is after 2, 4, 6, 8, 10 and 12, days. The metal extraction was estimated by Spectrophotometric methods described earlier.

4] Effect of Substrate Concentration on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*

The culture was grown under sterile condition in 250 ml Erlenmeyer flasks on a rotary shaker (140 rpm) and incubated at 32 ± 2 ^{0}C. After four days of incubation, the coal fly ash was added in the flasks with various concentrations such as 2, 4, 6, 8, 10 and 12 gm/100 ml. In control flask inoculum was replaced by a 40 ml of 9K medium along with 60 ml of sterile distilled water. Experiment was run for one month. The content of each Erlenmeyer flask was filtered through whatman no.41 filter paper and the filtrate was analyzed for the extracted amount of aluminum, iron, and manganese as per spectrophotometric methods described as earlier.

B) By *Pseudomonas fluorescens:*

The culture of ***Pseudomonas fluorescens*** was grown under sterile condition in 250 ml Erlenmeyer flasks on a rotary shaker (140 rpm) and incubated at 32 ± 2 ^{0}C after incubation of 24 hours. Pre weighed coal fly ash was added in Erlenmeyer flask such as 2, 4, 6, 8, 10 and 12 g/100 ml. Before use, the ash was washed with water to remove water soluble compounds and dried on a hot plate. In control flask, inoculum was replaced by a 40 ml of Kings Medium along with 60 ml of sterile distilled water. Experiment was run for one month. At the end of each experiment the final pH was measured after of the leaching experiment. The content of each Erlenmeyer flask was filtered through whatman no.41 filter paper. The metal extraction was measured by spectrophotometric methods described earlier.

N] MOLECULAR STUDIES OF METAL EXTRACTING MICROBES:

1] Studying Location of Metal Extraction Genes by Plasmid Curing:

A) In *Thiobacillus ferrooxidans*:

In order to find out whether bioextracting or bioleaching genes are located on genomic DNA or on plasmids, the plasmid curing experiment was carried out. Curing of *Thiobacillus ferrooxidans* was done according to method employed by Gohar et al. (2006). Ethidium bromide and acridine orange were used as curing agents. *Thiobacillus ferrooxidans* was grown in 40 ml Erlenmeyer flasks containing 9K medium was autoclaved at 121 ^{0}C for 15 minutes to prevent interference of other microorganisms and pH was adjusted to 2.0 with 10 N H_2SO_4. Each flask received the concentrations 25 µg/ ml ethidium bromide and 20 µg/ml of acridine orange. *Thiobacillus ferrooxidans* was grown in 20 ml flask was incubated at 32 ± 2 ^{0}C and sub cultured three times in 9K broth and incubated each time for 5 to 7 days. The 9K broth after three successive sub culturing was diluted and plated on the 9K agar plates. Well grown *Thiobacillus ferrooxidans* colonies were picked up and multiplied in 9K broth and the culture was used as inoculum for the bioextraction of metals from bauxite ore.

Bioleaching experiment was carried out to evaluate the bioextraction activity of cured and non-cured strains of *Thiobacillus ferrooxidans*. The experiment was carried out in Erlenmeyer flask containing 40 ml of 9K broth medium inoculated with 2 % concentration of bauxite ore. The bioleaching flask received 60 % of cured inoculum of *Thiobacillus ferrooxidans* (maximum biomass concentration 2.0 x 10^6 cells/ml). The flask containing 9K medium was sterilized by autoclaving at 121^{0}C for 20 min to avoid interference of indigenous microorganisms. It was then incubated for 30 days at 36 ^{0}C ± 2 ^{0}C at constant 140 rpm shaking condition. To compare

with non-cured *Thiobacillus ferrooxidans* same conditions were maintained. At the termination of experiment, the mixture was filtered and filtrate was analyzed for metal detection using method employed by Raquel et al. (2008) for aluminum and the method of Demirhan and Fikriye (2007) was employed for iron estimation.

B) In *Pseudomonas fluorescens*:

In order to find out whether metal bioextracting genes are located on genomic DNA or on plasmids, curing was carried out. ***Pseudomonas fluorescens*** was subjected to curing with ethidium bromide and acridine orange (Gohar et.al., 2006) ***Pseudomonas fluorescens*** was grown in 40 ml King's broth 25µg/ml each of ethidium bromide and acridine orange was added in tubes. Kings broth tubes inoculated with ***Pseudomonas fluorescens*** were incubated at 32 ± 2 ^{0}C and sub cultured three times in the broth tubes and incubated each time for 5 to 7 days. The King's broth, after three successive sub culturing, was diluted and plated on the King's agar plates. Well grown ***Pseudomonas fluorescens*** colonies were picked up and multiplied in king's broth and the inoculum was used for the extraction of metals from bauxite ore further.

Bioleaching experiment was carried out to evaluate the extraction activity of cured and non-cured strains of **Pseudomonas fluorescens**. The experiment was carried out in Erlenmeyer flask containing 40 ml of King's medium inoculated with 2 % loaded concentration of bauxite ore and bioleaching flask received 60 ml of cured inoculum of ***Pseudomonas fluorescens*** (maximum biomass concentration 2.0 x 10^4 cells/ml). It was then incubated for 21 days at 32 ± 2 ^{0}C at constant 140 rpm shaking condition. To compare with non-cured ***Pseudomonas fluorescens*** same condition was maintained. At the termination of experiment, the suspension was filtered and filtrate was subjected to the analysis of metals. Metal analysis was done as described earlier.

2] Genetic Profiling of *Thiobacillus ferrooxidans* and *Pseudomonas fluorescens* Mutants using ISSR Markers:

a) The Genomic DNA Isolation

Genomic DNA was extracted from 4 individuals. Two mother strains (*Thiobacillus ferrooxidans* and **Pseudomonas fluorescens**) and two mutants (*Thiobacillus ferrooxidans* mutant UV-26 and ***Pseudomonas fluorescens*** mutant UV-24) according to standard protocol with necessary modifications. Isolation of genomic DNA from bacterial cell was carried out using modified method published by Laboratory of Microbial Ecology, Department of, Earth, Ecological and Environmental Science, University of Toledo.

I) Preparation of Buffers and Chemicals

Lysis Buffer

- 400 mM Tris HCl (pH 7.5)
- 60 mM Na EDTA (pH 8.0)
- 150 mM NaCl
- 1% SDS

3M Sodium Acetate (pH 5.2)

- 40.83 gm of Sodium Acetate
- 80 ml of Distilled water
- The pH was adjusted to 5.2 and final volume was made to 100 ml.

TAE (Tris-Acetate-EDTA)

- Tris base 48.4 g
- Acetic Acid 11.42 ml
- 0.5 M EDTA 20 ml

The pH was adjusted to 8.5 using KOH and final volume was made upto 1 liter.

TBE (Tris-Borate-EDTA) (10X)

- Tris base 108 gm
- Boric Acid 55 gm
- Na EDTA 9.3 gm

The pH was adjusted to 8.3 using KOH and final volume was made upto 1 liter.

Ii) Protocol Used

The DNA was isolated from the two isolates; the isolates were grown in Luria Bertani broth overnight at room temperature. After incubation, 2ml of the broth was centrifuged at 10,000 rpm for 2 min to get the pellet. Overnight grown cells in 100 ml broth medium were used. The supernatant was removed and the pellet cells were resuspended in 1 ml of 50 mM Tris (pH 8.0), and 50 mM EDTA. Cell suspension was freezed at -20 ^{0}C and 200 µl of 250 mM Tris (pH 8.0), 50 µl of 10 mg/ml lysozyme to frozen suspension, and let thaw at room temperature. when thawed, place on ice for 45 min. 100 µl of lysis buffer was added followed by 1 mg/ml proteinase K. and whole assembly were placed in 50°C water bath for 60 min. The extraction was done with 1 ml phenol and centrifuged at 10,000 rpm for 15 min. Top layer was transferred to new tube to avoid interface. This step was repeated once. 0.1 volume of 3M Na acetate was mixed gently with 2 volume 95% ethanol and then mixed by inverting. The DNA was spooled out and transferred to new tube containing 50 mM Tris (pH 7.5), 1 mM EDTA, and 200 g/ml RNase. It was dissolved by rocking/vortexing at 4°C. Extraction was done with equal volume chloroform and mixed by inverting and centrifuged at 10,000 rpm for 5 min. The top layer was transferred to a new tube. 0.1 volume 3M sodium acetate was mixed gently with 2 volume 95% ethanol and mixed by inverting. The DNA was spooled out and dissolved in 200 µl of 50 mM Tris (pH 7.5) and 1 mM EDTA). The purity of DNA was checked by electrophoresis. The DNA pellet were dried and dissolved in TE buffer. The pellet was centrifuged at 5000 rpm for 15 minutes, drained and washed with 70% ethanol. The pellets were dried and dissolved in 100 µl TE buffer. The stock solution was stored at – 20^{0}C and the working solution at 4°C. A small aliquot of isolated DNA was run on a 1% (w/v) TAE gel to check the quality of DNA sample.

B) Quality Check of Isolated DNA

For checking the quality of isolated DNA, 2 µl DNA was diluted with 7 µl of TE buffer. After adding 1 µl 6x loading dye, 10 µl sample was loaded in a slot of 1% agarose gel containing 0.05 µg/ml of ethidium bromide. The agarose gel electrophoresis was carried out for nearly one hour at 50mA. The gel was visualized and photographed by gel documentation system. The intact double stranded DNA forming a thick single band of high molecular weight confirmed the good quality of genomic DNA extracted.

C) ISSR Marker

Inter Simple Sequence Repeats (ISSR), a molecular technique, was used for the assessment of genetic variability of two mother progenies and two UV-mutants.

ISSR Primers - ISSR primer sets were ordered from University of British Columbia (UBC). Total eight ISSR primers were used for initial screening. Out of eight primers, four primers gave

the amplification (in terms of repeatability, scorability) and were selected for identification as well as genetic variability in between mother progenies and UV-mutants.

The selected four primers were 14-23 mers based with various di - tri nucleotide SSR repeats.

Following primers gave the amplification. The primer sequences were as follows.

Primer 861- ACC ACC ACC ACC ACC ACC

Primer 862 - AGC AGC AGC AGC AGC AGC

Primer 867 - GGC GGC GGC GGC GGC GGC

Primer 868 - GAA GAA GAA GAA GAA GAA

D) PCR Amplification of DNA with ISSR Primers

PCR technique has promoted the development of a range of molecular assay systems which detect polymorphism at molecular level. In this study the most widely adopted PCR based ISSR marker technology was used for characterized the genetic variability amongst mother progenies and two UV based mutants. PCR reactions were carried out in a Finnzyme make thermal cycler.

I) Preparation of Master Mix for PCR

Taq buffer (10X)	- 2.0µl
DNTP's (10mM)	- 2.0µl
Taq polymerase (3U/ul)	- 0.4µl
Tween 20	- 0.2µl
MQ water	-3.4µl
Primer (3pmol/ ul)	- 2.0µl
Template DNA	-3.0µl

Ii) PCR Program Used

- 94 ^{0}C for 3 min - Hot start Denaturation
- 94 ^{0}C 15 sec – Denaturation (38 cycles)
- Annealing for 30 sec – temperature vary according to primer
- The reaction for 30 to 38 cycles.
- 72 ^{0}C for 1 minute – Extension (38 cycles)
- Last 72 ^{0}C for 5 minutes – final extension.
- Proceed 38 reactions
- After completing 38 cycles, samples were loaded on 1% Agarose gel in TBE. **Iii) Agarose gel electrophoresis**

The PCR products were separated by using 1% agarose gel in 1X TBE buffer.

Iv) Protocol Used

The 0.8g agarose was dissolved in 100ml of 1X TBE by gentle heating on magnetic stirrer with hot plate. Gel was prepared by pouring hot agarose in agarose gel tray situated in agarose gel apparatus supplied by Bangalore Genei Pvt. Ltd. The comb was inserted in such a way that 1mm gap between the teeth and surface of tray could be made easily and this was held for about 30-40 minutes. After solidifying the gel, the comb was removed gently. The 1x TBE buffer was poured into the buffer tank so as the gel should be immersed about 5mm in this buffer. The 3 µl amplified DNA samples and 3 µl of 6X gel loading buffer(0.25% bromophenol blue, 0.25% xyline cyanol FF, 20% glycerol in deionised water) were added and spun for 2-5 seconds.

Samples were gently loaded into the wells using microsyringe and run at 60 V until samples migrated to end of the gel. Gel was carefully removed and kept for staining.

V) Gel Staining and Documentation

1mg/ml ethidium bromide staining solution was used and the gel was photographed using Alpha Imager gel documentation system

Vi) Band Scoring and Data Analysis

For each sample, each fragment / band that was amplified using ISSR primers was treated as a unit rearrangement in genome. The primers which gave scorable and consistently reproducible amplicons were considered. The gel pictures were taken and documented to computer by using Alpha Imager gel documentation system and size of each amplicon was measured by using Alpha Imager Software with respect to standard molecular weight DNA ladder and the molecular weight of each of the potential specific band was calculated using the software program Alpha Imager.

Vii) Phylogeny Analysis

The Dendrogram was plotted by using bioinformatics phylogeny Free Tree and Tree View of DNA fingerprint analysis tool. Distance Similarity Matrix was calculated by using Neighbor-joining tree construction method of Nei and Li (1979) Dice index.

3] Protein Profiling by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE):

In order to study the protein profile at the time of metal extraction the proteins were isolated from the bacteria engaged with metal extraction. The water soluble proteins were analyzed by using the vertical slab gel electrophoresis apparatus. In SDS-PAGE, polypeptides were separated according to their molecular weight, and not by intrinsic electrical charge. Sodium dodecyl sulphate (SDS) is an anionic detergent that denatures proteins by wrapping around the polypeptide backbone. In doing so, the SDS confers a negative charge to the polypeptide in proportion to its length. When proteins are treated with SDS, the polypeptides become rods of negative charges with equal charge unit per length. Here, denaturing discontinuous PAGE system was used as described by Laemmli (1970). This system is almost similar to the native PAGE except for the presence of SDS.

A) Preparation of Solutions

I) Sodium Dodecyl Sulphate (10 %)

This was prepared by dissolving 20 g of SDS (anionic detergent) in 150 ml of distilled water and the final volume 200 ml was adjusted with distilled water.

Ii) Monomer Solution (Acrylamide/Bisacrylamide 29.9 % T, 0.9 % C)

30 g of acrylamide and 1.04 g of N, N-methylenebisacrylamide were dissolved in distilled water and made up to 100 ml.

Iii) Resolving Gel Buffer (1.5 Tris, Ph 8.8)

This solution was prepared by dissolving 18.16 gm Tris base in 60 ml of distilled water and the pH was adjusted with 0.1N HCL. The final volume was made upto 100 ml with distilled water and stored at 4^0C.

Iv) Stacking Gel Buffer (1 M Tris-HCL, Ph 6.8)

12.12 g of Tris was dissolved in 60 ml of distilled water. The pH 6.8 was adjusted with 0.1N HCL and the final volume was made upto 100 ml with distilled water and stored at 4^0C.

V) Stacking Gel Solution:

12ml stacking gel buffer, 17ml acryl bisacrylamide final volume made up to 100ml with double distilled water.

Vi) TEMED (10 %) Initiator

It was prepared by adding 100 µl TEMED to 900 µl of distilled water.

Vii) Ammonium Persulpahte (10 %)
100 mg of ammonium persulpahte was dissolved in 1 ml of distilled water. This solution was prepared freshly at the time of use.

Viii) Electrode Buffer (0.25 M Tris, 2 M Glycine, Ph 8.3)
The electrode buffer stock solution was prepared by dissolving 15 g of Tris base, 72 g of glycine in 300 ml of distilled water and then made to the final volume of 500 ml with distilled water. The pH of solution was adjusted to 8.3. This solution was diluted 10 times at each time of use.

Ix) Sample Buffer (0.08 M Tris-HCL, Ph 6.8)
1.6 ml stacking gel buffer was added to 2.5 gm of 87 % glycerol and 0.5 mg of bromophenol blue and mixed thoroughly and the final volume was made upto 10 ml with distilled water. It was stored at 4 ^{0}C.

X) Fixing Solution
57 gm trichloroacetic acid and 17 gm sulphosalicyclic acid were dissolved in 150 ml methanol and final volume was made upto 500 ml with distilled water.

Xi) Staining Solution
The staining solution was prepared by dissolving 1.25 gm coomassie brilliant blue R-250 in 230 ml of methanol and 40 ml of acetic acid, the volume was made upto 500ml with distilled water.

Xii) Destaining Solution
40 ml acetic acid and 230 ml ethanol were mixed well and made to the final volume of 500ml by adding distilled water.

B) Preparation of Resolving Gel
12 % polyacrylamide gel was prepared by mixing 12ml monomer solution, 10.5ml distilled water, 7.5ml resolving gel buffer,200 µl APS ,20 µl TEMED and 300 µl 10% SDS. Mixed the solutions and immediately this gel solution was poured into gel mould and over layered with water saturated n-butyl alcohol and allowed to polymerize. This resolving gel was prepared in a Broviga vertical slab gel plates.

After polymerization the n-butyl alcohol was poured off and the top of the gel was carefully washed with distilled water and later drained with paper strip.

C) Preparation of Stacking Gel
In 12 % of stacking gel solution, 50 µl APS, 10 µl TEMED and 50 µl 10% SDS were added and mixed thoroughly. This solution was quickly poured on top of the polymerized resolving gel. A comb was placed to form the sample well and the gel was allowed to polymerize. After polymerization the comb was removed carefully. The glass plates with the gel were fixed in the apparatus filled with the electrode buffer. Equal protein sample (40 µl) mixed with 2 x loading dye (sample buffer in 1:1 v/v) ratio were loaded in the sample wells. Electrophoresis was run at 150V, until the tracking dye came at end of the gel. The gel was fixed in 10 % TCA for 30 minutes. Then the gel was stained with staining solution for 5 hrs and washed briefly in destaining solution till the background became clear. The gel was photographed and molecular weight of every band was determined using gel documentation system.

D) Growth Condition in Presence of Metal Ions.
In the present investigation two bacterial mutants of *Thiobacillus ferrooxidans* (UV-mutants 26) and ***Pseudomonas fluorescens*** (UV- mutants 24) were used to detect the response to various metal ions or how bacteria interact with metals. *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** were grown in its respective media. Kings medium for *Pseudomonas* and 9K broth for *Thiobacillus* at 30 ± 2^{0}C and 32 ± 2^{0}C temperature. Metal ions were inoculated in the range

of 100µg/ml and 200µg/ml of each metal salts such as ferrous sulphate, aluminum potassium phosphate, titanium solution (Merck) and silicon chloride, whereas un-inoculated served as control for Pseudomonas as well as Thiobacillus mutants. 24 hours grown cells were harvested by centrifugation at 5000rpm for 10 min. and used for further studies.

E) Preparation of Cell Extract

The pellets were re-suspended and washed three times in double distilled water. Bacterial cells were disrupted by sonication with a sonicator two times for 30s with 1 min on ice in between. Samples were vortexed thoroughly, and shaken. Cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4^0C and supernatant was used as sample for SDS-PAGE analysis.

F) Application of Sample

10 µl 10% SDS was added to protein sample (90 µl) and mixed with 100 µl loading dye (containing 1% SDS, 25% glycerol, 0.1% 2-mercaptoethanol and 100mg bromophenol blue). This sample was heated in boiling water for 5 minutes and then used for electrophoresis. Electrophoresis was performed at constant voltage (150V) for 4.5 hours. The gels were fixed in 10 % TCA for 30 minutes. Then the gels were stained with staining solution for 5 hrs and washed briefly in destaining solution till the background became clear.

G) Determination of Molecular Weight of the Proteins:

The gels were photographed and molecular weight of every band was determined using gel documentation system. The Gene snap software was used for molecular weight determination.

O] FTIR STUDIES OF METAL EXTRACTING MICROBES

The samples were prepared by using Nujol mull and potassium bromide methods. To reduce IR absorption, the sample was placed in between windows of ionic material, potassium bromide.

In nujol Mull technique the sample was ground using a mortar and pestle to give a very fine powder. A small amount was then mixed with nujol to give a paste and several drops of this paste were then applied between two sodium chloride plates (these did not absorb infrared in the region of interest). The plates were then placed in the instrument sample holder ready for scanning.

In Potassium Bromide disk technique, a very small amount of the sample was (approximately 1-2 mg) added to pure potassium bromide powder (approximately 200 mg) and ground up as fine as possible. A disk of this powder was made with the help of small dye putting under pressure mechanically. The pressure was maintained for several minutes before removing the dye and the KBr disk formed. The disk was then placed in a sample holder ready for scanning. The samples were dried sufficiently before preparation.

Fourier transmittance infrared analysis was performed with Nicolet FTIR -740 instrument. The spectrum of *Thiobacillus ferrooxidans* mutant UV-26 and ***Pseudomonas fluorescens*** mutant UV-24 interacted with bauxite ore and fly ash was recorded. The filtered cell mass with bauxite ore and fly ash was collected dried at room temperature and used for IR measurements. For untreated, directly bauxite ore and fly ash was used for the IR measurements.

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EXPERIMENTAL RESULTS

A] PHYSICO CHEMICAL ANALYSIS OF METAL SOURCES:

1] Sample Collection and Physico – Chemical Analysis of Bauxite Ore and Fly Ash:

Table 3 indicates the physico-chemical properties of fly ash and bauxite ore. The texture of bauxite ore and fly ash was nearly same. The remarkable difference was noted in water holding capacity. The elements of both the metal sources were estimated in the form of total carbon, total nitrogen, phosphorous, potassium and sodium. The value of nitrogen and carbon content were differed by twice and six times more in bauxite as compare to fly ash. But the sodium content was found as nearly similar. Phosphorous content was found nearly same in both the metal sources. There was no much difference in the pH; however the pH of the fly ash was slightly alkaline. The porosity was found more in bauxite as compared to fly ash.

2] Mineralogical and Elemental Analysis of Bauxite Ore and Fly Ash:

The XRD analysis results of bauxite ore revealed the following phases, Diaspora, Kaolinite, Gibbsite, Hematite, Goethite, Boehmite, Trydymite-2OHsyn, Albite-disordered, Calcite, biotite-2M#1, Illite2M#2, Wallastonite, and Anatase (Table 4 and 6). The XRD pattern of bauxite ore is shown in Fig. 1.

The experimental results of XRD analysis of fly ash shown in Table 5 and 7 indicates that the main constituent of fly ash was Sodium aluminum silicate, Quartz, Coesite, Tricalcium manganese orthosilicate, Magnesium silicate, Calcium aluminum silicate, Ferric oxide, Mullite, Magnetite, and Hematite. The XRD pattern of fly ash is as per the Fig.2.

3] SEM and EDX Analysis of Bauxite Ore and Fly Ash:

EDX analysis stands for Energy Dispersive X-ray analysis. It is a technique used for identifying the elemental composition of the specimen. EDX analysis system works as an integrated feature of scanning electron microscope (SEM). From the bauxite ore the elements Al, Si, Ti, and Fe were analyzed. Tables 8 and 9 show the mass percentage of 46.27, 2.40, 1.46, 3.00 and 46.87 respectively. Atom percentage of these elements was also estimated as 35.62, 1.78, 0.63, 1.12 and 60.86 respectively. The mass percentage of aluminum was found maximum in bauxite while in fly ash iron was more in mass percentage. The atom percentage was found maximum in bauxite while in fly ash it was reported in case of Si. The energy was estimated as 1.486, 1.739, 4.508, 6.398, 5.894 and 1.253 in case of Al, Si, Ti, Fe, Mn and Mg respectively (Fig. 3, 4 and plate I and II).

4] Chemical Analysis of Bauxite Ore and Fly Ash:

Chemical constitution of bauxite ore indicated the more aluminum content than that of iron while in fly ash aluminum was reported as major component. Iron was found less than half of the aluminum content while manganese was found very less (Table 10 and 11).

B] MICROBIOLOGICAL ANALYSIS OF BAUXITE ORE AND FLY ASH

The study of microflora of bauxite ore at various depths of mines was made. The results are shown in Table 12. The microbial occurrence was different at different depths. Aspergillus flavus, *Aspergillus fumigatus, Fusarium oxysporum, Penicillium sp. Rhizopus stolonifer* were reported in all the five levels of depths (up to 20 m). *Aspergillus niger* was reported up to level 4. The fungi *Aspergillus kanagwensis* and *Aspergillus nidulans* were limited up to level 2. *Trichoderma* sp. and *Aspergillus terreus* were absent at the level 1. The relative frequency of *Aspergillus flavus* was found maximum in all the five levels of the depth. *Aspergillus fumigatus, Aspergillus niger, Fusarium oxysporum, Penicillium* sp. showed moderate relative frequency while *Aspergillus kanagwensis, Curularia lunata, Neocosmophora vasinpicta*, and *Trichoderma* and *Aspergillus nidulans* showed minimum relative frequency. The population of fungi was found decreased progressively with the increasing depth (Table 13) and frequency of

bacteria and actinomycetes was calculated as 36 x 10^2 and 42 x 10^2 for bauxite while in fly ash it was 21 x 10^2 and 8 x 10^2 (Table 14).

C] ISOLATION AND CHARACTERIZATION OF METAL EXTRACTING MICROBES FROM BAUXITE ORE:

1] Morphological and Biochemical Characteristics of *Thiobacillus ferrooxidans* Isolated from Bauxite Ore:

Thiobacillus ferrooxidans was found as gram negative rods of size 0.4 – 0.6 µm non-motile, with no sporulation. The metal extracting bacterium *Thiobacillus ferrooxidans* isolated from the bauxite ore was analyzed biochemically by performing various tests as per the Table 15. It was grown on ferrous iron as well as thiosulphate. This showed the autotrophic growth. Chemoorganotrophic growth of *Thiobacillus ferrooxidans* on glucose was also observed and the result was found positive. The bacterium was found resistant to the broad range of antibiotics. The pH of the medium 1.8- 2.0 gave luxuriant growth of *Thiobacillus ferrooxidans*. The critical tests for *Thiobacillus ferrooxidans* like catalase, starch hydrolysis, growth on thiosulphate were reported positive while the tests of the oxidase, citrate utilization, methyl red, urease, indole production and Voges Proskuer were found negative.

2] Morphological and Biochemical Characteristics of *Pseudomonas fluorescens* Isolated from Bauxite Ore.

The morphological dimension was found from 1-4 µm long gram negative rods. For the identification of ***Pseudomonas fluorescens*** the biochemical tests were conducted and the results are shown in Table 16. The ***Pseudomonas fluorescens*** was confirmed with the positive tests like glucose fermentation, catalase, oxidase, citrate utilization, however, the indole formation, nitrate reduction, urease formation, gelatin hydrolysis and maltose utilization were negative. Fluorescence of ***Pseudomonas fluorescens*** was tested under UV light after the growth on King's medium (Plate IV).

3] Effect of Ph on the Growth of *Pseudomonas* and *Thiobacillus* Isolates.

Effect of pH on the growth of ***Pseudomonas fluorescens*** and *Thiobacillus ferrooxidans*, isolated from bauxite ore was studied at various pH. The pH of the medium was adjusted with 10N H_2SO_4 and NaOH. It was seen that pH 4.0 was inhibitory for the growth of Pseudomonas isolates (Table 17) however, the growth was seen at the range of pH 5 to pH 10. The pH 7 to 8 was found to be highly favourable for the growth of Pseudomonas isolates whereas the Thiobacillus isolates showed controversial growth. Growth at acidic pH 1.5 to 2.5 was significantly suitable but pH 7 was inhibitory.

4] Growth of *Pseudomonas* and *Thiobacillus* Isolates at Different Nacl Concentrations.

The salt tolerance was remarkable in both the bacteria ***Pseudomonas fluorescens*** and *Thiobacillus ferrooxidans*. NaCl was incorporated in the King's as well as 9K liquid medium at the concentrations ranging from 0.5 to 5.0 percent. Results in the Table-18 show that Pseudomonas and Thiobacillus isolates could tolerate NaCl up to 4.5 percent only while 5.0 percent was inhibitory.

5] Growth of *Pseudomonas* and *Thiobacillus* Isolates at Different Temperature.

Effect of various temperatures ranging from 10^0C to 50^0C was studied and results are given in the Table-19. There was maximum growth of ***Pseudomonas fluorescens*** at 30^0C and 35^0C; whereas in *Thiobacillus ferrooxidans* 30^0C to 40^0C was found most suitable.

6) Oxidation Rate of Ferrous IONs in Presence of Bauxite Ore and Fly Ash by *Thiobacillus ferrooxidans*:

The oxidation rate of ferrous ions in presence of bauxite ore and fly ash by *Thiobacillus ferrooxidans* increased with the increased incubation time, but as compare to control slight

decrease in the curve occurred (Table 20 and Graph 01). It is due to the inhibition of metals which residing in metal sources.

7) Growth of *Pseudomonas fluorescens* in Presence of Bauxite Ore and Flyash

Growth curves of ***Pseudomonas fluorescens*** in presence of bauxite ore and fly ash showed the constant increase upto 26 and 36 hrs for bauxite and fly ash respectively. Afterwards there was decline phase. In presence of bauxite and flyash ***Pseudomonas fluorescens*** extended the log phase. It is due to availability of these metal sources. Delaying of log phase is because of adaptation time required for the bacterium (Table 21 and Graph 02).

8) Effect of Various Metal ION Concentrations on Growth of *Pseudomonas fluorescens* and *Thiobacillus ferrooxidans*:

In the present investigation, effect of various metal ions (titanium, silicon, aluminum and iron) were studied and found that both the bacteria *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** were very much resistance to metal ions. Metal salts of aluminum, silicon, titanium and iron in the range of 1mg/ml to 5mg/ml were added and un-inoculated served as control. Results (Table 22, 23 and Graph 3, 4) indicated that the ***Pseudomonas fluorescens*** tolerated upto 3mg/ml. Above this concentration it showed decline in growth. However, *Thiobacillus ferrooxidans* was highly resistance to all metal concentration but 3mg/ml showed maximum growth.

D) BIOEXTRACTION OF METALS FROM BAUXITE ORE:

1] Comparative Metal Extraction by Adapted and Non Adapted *Thiobacillus ferrooxidans* Strains:

Results are presented in Table 24. It was found that the adapted strain of *Thiobacillus ferrooxidans* was efficient in metal extraction as compare to non-adapted strain. 79.48 percent aluminum was extracted by adapted strain while 74.54 percent by non- adapted one. However, in iron extraction difference was remarkable. Adapted strain extracted 81.78 percent while non-adapted, extracted only 69.10 percent. When aluminum and iron considered together same trend was observed.

2] Metal Extraction by Adapted and Non Adapted Strains of *Pseudomonas fluorescens*:

Results are presented in Table 25. It was found that the adapted strain extracted more metal as compared to non- adapted, but the difference was not remarkable. Same results were obtained when bioextraction efficiency of two metals was considered.

E) GENETIC IMPROVEMENT OF METAL EXTRACTING MICROBES:

1] Induction of Mutation into the Metal Extracting Microbes ay Physical and Chemical Mutagens:

The mutants of ***Pseudomonas fluorescens*** and *Thiobacillus ferrooxidans* were developed by physical (UV) and chemical (EMS) mutagenic agents. The results of mutagenesis indicated that, as the concentration of the chemical mutagenic agent, EMS was increased the number of mutants colonies were found reduced in both the microorganisms. Similarly when UV radiation was used the mutation frequency was reduced. This is because of the damaging effect of mutagenic treatment. Due to excess dose of mutagens the DNA of microbes get damaged to that extent in such a way that it never get repaired and it leads to the death of the organism, such dose of treatment called as lethal dose (Muller, 1995). Half lethal dose is known as LD_{50}. For ***Pseudomonas fluorescens*** LD_{50} of EMS treatment was found to be 13.01 mg/ml. And LD_{50} of UV exposure was calculated as 10.2 minutes for *Thiobacillus ferrooxidans* the LD50 of EMS and UV treatment were found to be 6.32 mg/ml and 6.52 minutes exposure respectively (Table 26 and Graph 5, 6).

F) EXTRACTION OF METALS FROM BAUXITE ORE BY GENETICALLY IMPROVED MICROBES:

5] Extraction of Metals Using Thiobaillus Ferrooxidans Mutants:

A) Extraction of Metals by UV- Mutants of *Thiobacillus ferrooxidans*.

Results are presented in Table 27. A total of 30 UV mutants were isolated from the plates and tested for bioextraction of metals from bauxite ore. Out of the 30 UV-mutants 14 mutants were found more efficient for aluminum extraction while 16 isolates were found to be more efficient for iron extraction. Out of the total 30 isolates of *Thiobacillus ferrooxidans* mutants induced by UV radiation the isolate UV- 26 showed maximum extraction of metals.

B) Extraction of Metals by EMS- Mutants of *Thiobacillus ferrooxidans*:

Results are presented in Table 28. It was noted that out of 30 mutants only two mutants (isolate-22 and isolate-26) showed more extraction of aluminum while 8 mutants were found to be more efficient for iron extraction.

C) Extraction of Metals by UV- Mutants of *Pseudomonas fluorescens*:

Results are shown in Table 29. From the UV-induced mutants of ***Pseudomonas fluorescens*** 24 isolates were obtained. The 7, UV- isolates, showed more bioextraction of iron while 5 isolates showed more extraction of aluminum. The isolates of ***Pseudomonas fluorescens*** UV mutants 20 and 24 gave maximum aluminum extraction. The mutants 16, 17, 20, and 21were found significant for iron extraction. It can be concluded that the induction of mutation by UV radiation was fruitful for increasing the metal extracting efficiency of **Pseudomonas fluorescens**.

D) Extraction of Metals by EMS-Mutants of *Pseudomonas fluorescens*:

Results are shown in Table 30. Out of 24 mutants of ***Pseudomonas fluorescens*** mutant-20, showed significantly maximum aluminum and iron extraction, whereas 8 mutants demonstrated more bioextraction of iron than that of control, while in case of aluminum the number was only 3.

G) EFFECTS OF ENVIRONMENTAL FACTORS ON BIOEXTRACTION OF METALS FROM BAUXITE ORE:

1] Effect of Media Composition on Extraction of Metals by *Thiobacillus ferrooxidans*:

Results are shown in Table 31and Graph 7. Aluminum extraction was higher in the medium 9k without glucose, followed by T and K and 9k with glucose, Marsh and Norris medium and basal salt medium. The iron extraction was found to be highest in 9k medium without glucose followed by T and K medium, basal salt medium and Marsh and Norris medium. Consequently the percentage bioextraction efficiency of aluminum and iron was noted to be higher in 9K without glucose (78.56 %) followed by T and K medium (75.21 %) and 9k with glucose, basal salt media and Marsh and Norris medium.

2] Effect of Ph on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:

The results in Table 32 and Graph 8 indicate that, the aluminum extraction ranged from 40.12 percent to 81.13 percent. The pH 2.0 gave higher extraction than pH 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 in the decreasing manner. In case of iron extraction pH 2.0 was highly effective and pH 7.0 and 8.0 were lest effective. This also corresponds to the percent iron extraction and bioextraction efficiency together.

B) By *Pseudomonas fluorescens*:

Results are presented in Table 33 and Graph 9. Aluminum extraction was noted to be increased upto pH 8.0 and thereafter there was decline in aluminum extraction from pH 9.0 to 10.0. In case of iron again the extraction was increased from pH 4.0 to 8.0 and thereafter there was

decline from 9.0 to 10.0. When both the aluminum and iron extraction considered together again pH 8.0 was found to be the best for bioextraction of these metals.

9] Effect of Temperature on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*

Results in Table 34 and Graph 10 indicate that with the increase in the temperature the capacity of aluminum extraction also increased upto 44 ^{0}C however the extraction was decreased at 48 ^{0}C. The extraction of iron also showed the same pattern. The temperature 36 ^{0}C showed higher extraction in comparison to the other temperatures. When aluminum and iron considered together, 36^{0}C temperature showed higher bioextraction efficiency.

B) By *Pseudomonas fluorescens:*

The results are presented in Table 35 and Graph 11. It was noted that the aluminum extraction increased upto 32 ^{0}C. Increase in temperature 36^{0}C to 48 ^{0}C showed decline in aluminum extraction. Iron extraction, however, was increased upto 32 ^{0}C and thereafter there was decline in extraction of iron. Bioextraction efficiency for aluminum and iron together also showed that 32 ^{0}C is most favourable for the metal extraction.

10] Effect of Residence Time Extraction of Metals:

A) By *Thiobacillus ferrooxidans*

It was seen that the bioextraction of metals was increased with the increase in incubation time. Table 36 and Graph 12 indicate that aluminum extraction was increased from 167.66 to 606.67 mg with the increase in incubation period. These also correspond to aluminum and iron extraction together. The iron extraction was also increased with the increase of incubation period. The extraction ranged from 86.34 to 286.23 mg/ 2g ore at the increase of 4 days and 40 days of incubation respectively.

B) By *Pseudomonas fluorescen*:

It was reported that with the increasing incubation period the extraction of aluminum was also increased. It rose from 3.22 percent at 2 days incubation to 40.01 percent extraction of aluminum for 20 days incubation period. Similar results were obtained in case of iron. It ranged from 3.75 percent at 2 days to 72.05 percent at 20 days of incubation. When both the aluminum and iron were considered it was seen that with the increase of incubation time bioextraction efficiency was also increased (Table 37 and Graph 13).

11] Effect of Substrate Concentration Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:

It was noted that 5 gm of the substrate (pulp) concentration gave more extraction when compared with other bauxite ore concentrations. In general 5 gm bauxite ore concentration was found to be better for the aluminum extraction. Iron extraction also ranged from 198.43 to 286 mg at 1- 4 gm of the bauxite ore. Iron extraction ranged from 171 (least) to 286 mg at 10 gm and 4 gm respectively. When both the aluminum and iron extraction efficiency was calculated, it was noted that it ranged from 45.74 to 81.60 at 1.0 gm to 4.0 gm substrate concentration respectively (Table 38 and Graph 14).

B) By *Pseudomonas fluorescens*:

Results in the Table 39 and 15 show that aluminum extraction was higher at 4 gm bauxite ore concentration. Other pulp density also gave aluminum extraction from 25.32 to 38.46 at12 and 2 gm bauxite ore concentrations respectively. Iron extraction was also found variable at different pulp densities. The maximum iron extraction was found at 2 gm and 4 gm. In other cases it was found to be 46.23 percent to 67.27 percent for 12 g and 6 g respectively. Bioextraction efficiency of aluminum and iron also shows that 2 gm and 4 gm were most favourable for extraction of metals from bauxite ore.

12] Effect of Agitation on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:

It was observed that aluminum extraction was increased with the increase of agitation speed. Same type of result was obtained for iron. Both the aluminum and iron extraction efficiency was calculated. It was seen that with the increase of agitation speed the extraction efficiency was also increased (Table 40 and Graph 16).

B) By *Pseudomonas fluorescens*:

Results in the Table 41 and Graph 17 indicate that with the increase of shaking speed from 0-140 rpm, aluminum extraction was increased. The extraction ranged from 19.55 to 40.65 percent. In case of iron, the extraction was increased from 36.99 percent (at 0 rpm) to 70.93 percent (at 140 rpm). Similar results were obtained when bioextraction of aluminum and iron considered together at 140 rpm and the bioextraction efficiency was increased from 24.96 percent (stationary state) upto 50.0 percent.

13] Effect of Particle Size on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:

Table 42 and Graph 18 explains that the aluminum and iron extraction was reduced with the increase in the particle size upto 66.29 and 43.90 (5-6 mm particle size) percent respectively. The efficiency of bioextraction was reduced to 59.34 percent due to increase in the particle size. Metal extraction efficiency was also decreased.

B) By *Pseudomonas fluorescens*:

The observations, after 30 days showed that the minimum size of particles gave maximum aluminum extraction (36.53 to 33.40 percent). In case of iron same type of results were obtained. Maximum extraction was found from 0.20 -0.40 mm and increase of particle size decline the iron extraction. When aluminum and iron were considered together the same trend was observed (Table 43 and Graph 19).

14] Effect of Inoculum Concentration on Extraction of Metals:

A) By *Thiobacillus ferrooxidans* Inoculum:

It was noted that with the increase of inoculum concentration, the aluminum and iron contents were increased. The extraction ranged from 60.30 to 81.31 percent for aluminum whereas percent iron extraction increased from 43.39 to 81.96 percent for iron. Bioextraction efficiency was ranged from 55.05 to 81.51 percent as shown in Table 44 and Graph 20.

B) By *Pseudomonas fluorescens* Inoculum:

Results are presented in Table 45 and Graph 21. It was noted that aluminum extraction increased with the increase of inoculum. At 100 percent inoculum 44.86 percent aluminum was extracted. Similar types of results were obtained in case of iron. Iron extraction was increased to 34.40 percent (at 10 percent inoculum concentration) to 67.47 percent (at 100 percent inoculum concentration).

15] Effect of Nacl Concentration on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:

Results are given in Table 46 and Graph 22. It was noted that aluminum and iron extraction was reduced with the increase of NaCl concentration. Aluminum extraction ranged from 22.43 (5 % NaCl) to 80.49 (0.5 % NaCl) concentration. In case of iron it ranged from 40.43- 87.87 % with the same concentration of NaCl. For both the metal extraction the efficiency of *Thiobacillus ferrooxidans* was decreased with the increasing concentration of NaCl.

B) By *Pseudomonas fluorescens*:
After 30 of incubation it was observed that aluminum extraction increased with the decrease of NaCl concentration. At 0.5 percent concentration, aluminum extraction was higher and then decreased to 11.26 percent at 5.0 percent NaCl concentration. Similar results were obtained in case of iron. Iron extraction was also found higher at 0.5 percent NaCl concentration (54.91 %) and decreased to 1.5 percent NaCl concentration (44.51 %). Results obtained with aluminum and iron extraction were more or less same (Table 47 and Graph 23).

H) EFFECT OF CELL METABOLITES ON BIOEXTRACTION OF METALS FROM BAUXITE ORE:

1] Using *Thiobacillus ferrooxidans* Cell Free Extract:
Results in Table 48 indicate that, with the addition of *Thiobacillus ferrooxidans* aluminum extraction was increased (74.90 percent) than that of by cell metabolites (36.02 percent). In the absence of bacterium only 56.29 percent iron was extracted. In case of iron, extraction was highly increased (81.83 percent) when compared with and without *Thiobacillus ferrooxidans* cells.

2] Using *Pseudomonas fluorescens* Cell Free Extract:
Results in Table 49 indicate that with the addition of ***Pseudomonas fluorescens*** cells aluminum extraction was increased (18.49 percent) than that of cell metabolites (in cell free extract) (13.31 percent). In the absence of bacterium only 21.99 percent iron was extracted. In case of iron extraction it was high (57.38 percent) in presence of cells while in cell free extract it was decreased (21.99 percent).

I] SYNERGISTIC EFFECT OF MICROBES ON BIOEXTRACTION OF METALS FROM BAUXITE ORE:

1] Combined Effect of Metal Extracting Microbes with Other Microbes:

A) *Thiobacillus ferrooxidans* and Other Bacteria**:**
Thiobacillus ferrooxidans when inoculated with other bacteria like **Pseudomonas fluorescens**, Pseudomonas aeruginosa, Bacillus cereus, Bacillus polymyxa and Bacillus megaterium individually in combination with bauxite ore it was found that aluminum extraction was increased with ***Pseudomonas fluorescens*** and Bacillus megaterium combination with *Thiobacillus ferrooxidans*. Iron extraction was increased by addition of all of these microbes. The efficiency of Thiobacillus for bioextraction of metals was found to 60.19 to 69.61 percent for aluminum and iron respectively (Table 50).

B) *Pseudomonas fluorescens* and Other Bacteria**:**
When ***Pseudomonas fluorescens*** was mixed with Pseudomonas aeruginosa, Pseudomonas cissicola, Bacillus polymyxa, Bacillus cereus and Bacillus megaterium, the combination of ***Pseudomonas fluorescens*** with Pseudomonas aeruginosa gave higher amount of aluminum extraction (43.74 percent) (Table 51). In case of ***Pseudomonas fluorescens*** 33.51 percent aluminum extraction was done. In case of iron, mixing of ***Pseudomonas fluorescens*** with Pseudomonas aeruginosa gave higher extraction (77.84 percent). In other cases there was decline upto 55.58 percent. The extraction was 51.52 percent in case of ***Pseudomonas fluorescens*** only. When aluminum and iron extraction was considered together it was seen that, ***Pseudomonas fluorescens*** in combination with Pseudomonas aeruginosa gave higher bioextraction efficiency (54.33 percent). But when only ***Pseudomonas fluorescens*** was used it gave 39.10 percent extraction.

C) *Thiobacillus ferrooxidans* and Fungi**:**
In the combine effect of *Thiobacillus ferrooxidans* and the fungi Aspergillus niger, Aspergillus flavus, Penicillium sp. Rhizopus sp the results obtained are shown in Table 52. It was noted that the addition of these fungi, in combination with *Thiobacillus ferrooxidans* resulted in decreased

aluminum extraction. Similarly iron extraction was also found reduced. *Thiobacillus ferrooxidans* in combination with Rhizopus arrihizus reduced iron extraction very greatly.

D) *Pseudomonas fluorescens* and Fungi**:**
When ***Pseudomonas fluorescens*** was mixed with the spores of Aspergillus niger, Aspergillus flavus, Penicillium sp. and Fusarium sp., only ***Pseudomonas fluorescens*** alone gave higher extraction of aluminum. In combination with these fungi the extraction was minimized. In case of iron extraction, mixing of ***Pseudomonas fluorescens*** with fungi the effect was inhibitory. Thus when both the metals aluminum and iron were considered, addition of fungi gave negative results (Table 53).

J] MICROBIOLOGICAL ANALYSIS OF FLY ASH:
The fly ash showed occurrence of Aspergillus niger, Aspergillus fumigatus, Penicillium sp., Aspergillus flavus, Penicillium notatum, Aspergillus terreus and Fusarium sp. Species were found within the range of 9-19 % while Aspergillus carboniferous and Helminthosporium sp. were occurred at 4.54 and 3.48 respectively. The bacterial population was found in 21 x 10^2 and actinomycetes were occurred at 8 x 10^2 colony forming units. (Table 54).

K] BIOEXRACTION OF METALS FROM FLY ASH BY GENETICALLY IMPROVED MICROBES:

1] Extraction by Adapted and Non- Adapted Mutant Strains of *Thiobacillus ferrooxidans*:
Table-55 shows that the adapted strain was dominant in bioextraction of metals in case of aluminum extraction which was noted to be 79.94 percent. Non adapted strain was less effective (66.02 percent) comparatively. Same results were obtained in case of iron and manganese.

2] Extraction by Adapted and Non-Adapted Mutant Strains of Pseudomonas fluorescens:
Results are shown in Table 56. It was observed that the adapted strain extracted more metal as compare to non-adapted one. Aluminum extraction increased up to 40.84 mg by adapted strain while in case of non adapted strain it was decreased up to 36.68 mg same types of results were obtained in case of iron and manganese. Bioextraction efficiency was found increased in case of adapted strain (25.41) and decreased in non adapted strain (22.84).

L] EFFECT OF ENVIRONMENTAL FACTORS ON BIOEXTRACTION OF METALS FROM FLY ASH:

1] Effect of Ph on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:
Results are presented in Table 57 and Graph 24. Aluminum extraction was noted to be increased upto pH 1.5 to 2.5 and thereafter there was decline in aluminum extraction from pH 3.0 to 8.0. In case of iron extraction again the extraction was increased upto pH 2.5 (88.04 percent) and thereafter there was decline from 4 to 8 but in case of manganese extraction pH 2 (71.42) was more favorable. When these three metals aluminum, manganese and iron considered together again pH 2 to 2.5 was found to be more suitable.

B) By Pseudomonas fluorescens:
Aluminum extraction was noted to be increased upto pH 8.0 (30.22 percent) and thereafter there was decline from pH 9 to 10 (16.32 percent). In case of iron extraction again the extraction was increased from pH 5 to 8 (40.86 percent) and thereafter there was decline from 9 to 10 (18.88 percent) (Table 58 and Graph 25).

2] Effect of Temperature on Extraction of Metals:

A) By *Thiobacillus ferrooxidans*:
The temperature play crucial role in the bioextraction of metals from fly ash. Results in Table 59 and Graph 26 indicate that, with the increasing temperature the aluminum extraction capacity

was also increased upto 38 ^{0}C and then decreased at 40 ^{0}C. The extraction of iron and manganese also showed the same pattern.

B) By Pseudomonas fluorescens:
Results are presented in Table 60 and Graph 27. It was noted that the aluminum extraction was increased upto 34 ^{0}C. Increasing temperature, 38^{0}C to 48 ^{0}C showed decline in aluminum extraction. Iron extraction was also found increased upto 34 ^{0}C and thereafter there was decline. In case of manganese extraction same trend was observed. Bioextraction efficiency of aluminum manganese and iron together showed that 34 ^{0}C is most favourable temperature for the metal extraction.

3] Effect of Residence Time on Extraction of Metals:

a) By *Thiobacillus ferrooxidans*:
Table 61 and Graph 28 indicate that aluminum extraction was increased from 3.74 percent to 60.46 percent with the increase of incubation period. This also corresponds with manganese and iron extraction. The iron extraction was increased with the increase of incubation period, (ranged from 2.34 to 87.36 percent at the increase of 2 days and 24 days respectively).

b) By Pseudomonas fluorescens:
With the increase in the incubation period there was increase in the extraction of aluminum. It rose from 1.72 percent at 4 days of incubation to 13.39 percent extraction of aluminum at 24 days of incubation period. Similar results were obtained in case of iron; it ranged from 6.64 percent at 4 days to 60.39 percent at 24 days. In case of manganese same type of results were obtained. When three metals, aluminum, manganese and iron were considered, it was seen that with the increase of incubation time bioextraction efficiency was also increased (Table- 62 and Graph 29).

4] Effect of Substrate Concentration Extraction of Metals:

a) By *Thiobacillus ferrooxidans* :
It was noted that 6 gm of the fly ash concentration gave more extraction when compared with other fly ash concentrations for the aluminum extraction. Aluminum extraction also ranged from 86.23 to 164 mg at 2 to 6 g of fly ash concentration. Iron extraction was ranged from 39.42 to 104.65 mg at 2 g and 6 g respectively. Same type of extraction pattern was observed in case of manganese. When all the three metals were considered together the metal extraction efficiency was calculated as 74.54 to 13.72 at 2gm to 12 gm substrate concentration respectively (Table-63 and Graph 30).

B) By Pseudomonas fluorescens:
Table 64 and Graph 31 shows that aluminum extraction was higher at 6 gm fly ash concentration. Other fly ash concentrations also gave aluminum extraction from 20.80 to 26.18 at 12 and 2gm. Iron and manganese extraction was also found at different fly ash concentration. The maximum iron extraction was found at 6 gm to 8 gm. In case of manganese it was found at 4gm.

M] MOLECULAR STUDIES OF METAL EXTRACTING MICROBES:

1] Studying Location of Metal Extraction Genes by Plasmid Curing:

A) In *Thiobacillus ferrooxidans*:
Results are presented in Table 65. The acridine orange and ethidium bromide treated cells and non-treated cells did not show any significant difference in the bioextraction of aluminum and iron. Hence it is confirmed that the genes responsible for metal bioextraction were not plasmid borne. These must be located on the genomic DNA.

B) In Pseudomonas fluorescens:

Results are presented in Table 66. The acridine orange and ethidium bromide treated cells and non-treated cells did not show any significant difference in the bioextraction of aluminum and iron. Therefore it is confirmed that the genes responsible for metal bioextraction were not present on plasmid. These must be residing on the genomic DNA.

2] Genetic Profiling of *Thiobacillus ferrooxidans* And *Pseudomonas fluorescens* Mutants Using ISSR Markers:

In the present study, out of 8 ISSR primers 4 primers exhibited polymorphism with the two mother progeny (*Thiobacillus ferrooxidans*, **Pseudomonas fluorescens**) and two mutants (*Thiobacillus ferrooxidans* UV mutant 26 and ***Pseudomonas fluorescens*** UV mutant 24) which are summarized in Table 65. Four ISSR primers detected a total of 57 amplified fragments, varying from 8 (UBC- 868) to 18 (UBC-867) fragments per primers and ranged from 270 to 1860 base pair in size. Amplification of genomic DNA of two mother progeny and two mutants such as UV mutant 26 and UV mutant 24 using ISSR markers yielded 57 fragments, of which 17 were polymorphic while the remaining were monomorphic in nature. All the tested primers revealed polymorphism among two mother progeny and two mutants ranging from 0 percent for primer UBC-868 to 42.85 percent for primer UBC-862. The overall polymorphism for the four primers across all (mutant and mother progeny) isolates was 29.82 percent. Ratio of polymorphic bands with monomorphic bands was calculated as for primer UBC-861 produced bands in the ratio of 1: 2.39. For primer UBC-862 it was 1: 1.33, for primer UBC-867 it was 1: 2.00 and for primer UBC-868 it was 1:0.00. For total the ratio 1: 2.35 was observed (Table 67, 68, 69,70,71,72 and 73) (Plate V and VI).

ISSR based dendrogram indicates (Fig-5) that the two bacterial species (*Thiobacillus ferrooxidans* and **Pseudomonas fluorescens**) and UV mutant 26 of *Thiobacillus ferrooxidans* and UV mutant 24 of ***Pseudomonas fluorescens*** used in the present study could form three clusters (cluster-I, II, III) having *Thiobacillus ferrooxidans* UV mutant 26, ***Pseudomonas fluorescens*** mother progeny and ***Pseudomonas fluorescens*** UV mutant 24 respectively.

Cluster I comprise of *Thiobacillus ferrooxidans* UV mutant 26 and mother progeny of *Thiobacillus ferrooxidans*. The cluster II carried mother progeny of **Pseudomonas fluorescens**. The cluster III comprised of only one ***Pseudomonas fluorescens*** UV mutant 24. The present results reported that ISSR technique gave adequate distinctions among mother progeny and mutants under investigation (Table 71).

3] Protein Profiling by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE):

To investigate a possible correlation between metal ions tolerance level and protein profile of the *Thiobacillus ferrooxidans* mutant UV-26 and ***Pseudomonas fluorescens*** mutant UV- 24 the total proteins were extracted and analyzed by SDS-PAGE. Mutants of Pseudomonas and Thiobacillus showed quite similar protein profile pattern when compared with un-inoculated control. The protein molecular weight ranged from 10.00 to 120.00 KDa (Table 74 and 75). The number of bands varied from 10 to 24. Hence it was concluded that in the presence of different types of heavy metals, mutants of *Thiobacillus ferrooxidans* UV-26 and ***Pseudomonas fluorescens*** UV-24 did not showed any significant difference in their protein profile pattern (Plate -VII).

N] FTIR STUDIES OF METAL EXTRACTING MICROBES:

The samples can be characterized as well as reaction products can be identified at various temperatures, a range of techniques are used, including wet chemical analysis, x-ray diffraction (XRD) and spectroscopic techniques such as Fourier Transform Infrared (FTIR) Spectroscopy.

An IR spectrum of a given compound is unique and can therefore serve as a fingerprint for the compound. Infra red spectroscopy works on the principle of absorption measurements of different IR frequencies by a sample positioned in the path of an IR beam. IR spectroscopy is an important and widely used tool for structural phase identification and compound identification.

IR absorption information is generally presented in the form of a spectrum with wave number as the x-axis and absorption intensity or percent transmittance as the y-axis.

The infrared region of the electromagnetic spectrum extends over wide range from 14,000 cm^{-1} to 10 cm^{-1}. The region of most interest for chemical analysis is the mid-infrared region (4,000 cm^{-1} to 400 cm^{-1}) which corresponds to changes in vibrational energies within molecules. The far infrared region (400 cm^{-1} to10 cm^{-1}) is useful for molecules containing heavy atoms such as inorganic compounds. This technique carries major advantages as, (i) a quick and relatively cheap spectroscopic technique, (ii) useful for identifying certain functional groups in molecules.

Fig. 6, 7, 8 and 9 for *Thiobacillus ferrooxidans* mutant treated with bauxite and fly ash along with untreated one as well as and Fig 10, 11, 12 and 13 for ***Pseudomonas fluorescens*** mutant treated with bauxite and fly ash along with the untreated one show two spectra of FTIR bearing wave number on X axis and percent transmittance on Y axis. The lower spectrum is of untreated bauxite ore while the upper spectrum is of bauxite ore after treatment of *Thiobacillus ferrooxidans* mutant UV-26. It was observed from the spectrum that, it can be divided into three regions first region is from 400 cm^{-1} to 600 $cm.^{-1}$. Second one is from 600 to 2400 cm^{-1} and the third one is form 2400 to 4000 $cm.^{-1.}$ The regions were labeled as a, b and c. Region a is the region crowded with many absorption bands. Region b contains regularly spaced absorption bands while region c contains only one major absorption band with a large shoulder. The band appeared at 1559 cm^{-1} in the untreated bauxite ore spectrum has disappeared in the spectrum of treated sample. Moreover it can be observed that some additional bands in region b at around 1202 $cm^{-1,}$ 1076 cm^{-1} and 1003 cm^{-1} appeared in the spectrum of treated sample. The percent intensity of bands has remained at around 35 to 45 % in the untreated spectrum which was reduced to a value of 15 to 30 % value in the treated sample spectrum. The bands in the lower side spectrum 300 to 700 cm^{-1} are assigned to crystal structural vibrations that occur in the oxygen and iron ion present in the sample. The bands when observed at around 1000 cm^{-1} and 1100 cm^{-1} then they can be assigned to O-H bending vibration. Absorption bands in the region 1100 to 1300 cm^{-1} are generally assigned to nitrate ions. The bands in the region of 1300-1400 cm^{-1} are attributed to the characteristic CH_3 bending, an absorption band corresponding to carboxyl group (COO-) are observed at 1500 cm^{-1} and absorption bands in the range of 3100 to 3450 cm^{-1} corresponds to Hydrogen bonded O-H groups. The peaks at 2800 and 2920 cm^{-1} are due to C-H stretching vibration.

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DISCUSSION

Biomining is generic term that describes metal containing ores using microbial technology. This is an alternative to traditional physical and chemical methods of mineral processing. Microbial leaching is a procedure that refers to the natural ability of microorganisms to solubilize mineral constituents of ores. Microbial leaching may be therefore manifesting itself in the extraction or mobilization of valuable metals from an ore and industrial wastes.

Presently bioleaching is the leading mineral processing technique where metals are dissolved from mineral ores into solution through oxidation and decomposition process of the ores by microorganisms followed by the extraction of these metals from the solution. The present work was undertaken to standardize a process which is most suitable for extraction of metals from mineral concentrate as well as industrial waste. The sources used in this work were bauxite ore, which was collected from Radhanagri bauxite mines, Kolhapur and the fly ash was obtained from Thermal Power Station, Parli. In the present investigation physicochemical analysis of bauxite ore and fly ash was done using XRD in which different mineralogical phases have been clarified (Ambreen et al., 2002; Ghorbani et al., 2007). SEM –EDX techniques as well as carbon, nitrogen, phosphorus, potassium and sodium were also analyzed. EDAX analysis showed that Aluminum was 46.27 % whereas iron mass was 46.87 % while other elements showed in less quantity. These results were supported by the work of Francis et al. (1999) who have detected chemical composition of bauxite ore. The mass percentage and energy was found in confirmation with Rashid et al. (2001).

The quality of the metal source like bauxite is determined with respect to its impurities mainly iron, the choice of process technology and the economics of production. Presently low iron bauxite usually preferred (Mittra et al., 2003). The bauxite showed 21.45 percent water holding capacity, pH 7.5 and colour was dark red. The nitrogen, phosphorus, potassium and sodium were found in trace but carbon was reported maximum (0.3 %). The fly ash analysis for different elements has also been studied. The carbon content within the fly ash was reported as 0.12 percent while nitrogen content within the fly ash was worked out 0.003 %. The phosphorus 0.035 % while potassium was estimated as 0.02 % (Mittra et al., 2003). When sodium is considered it was found that 0.123 mg/g which is supported by Juwarkars et al. (1992). Phosphorous content was found nearly same in both the metal sources and found within the range given by earlier workers.

The pH of the bauxite was found almost neutral (Choudhary, 2007) During the literature survey it was found that no much difference in the pH was observed, however the pH of the fly ash was slightly alkaline. Similar results were obtained by Mohapatra and Kanungo (1994), who reported during physiochemical characteristics of fly ash samples from thermal power plants of Orissa.

The presence of microorganisms in the bauxite ore and fly ash ore were also studied. Bauxite ore indicated the microbes occurred at different depths. Aspergillus flavus, Aspergillus fumigatus, Fusarium, oxysphorum and Rhizopus present at all levels whereas Aspergillus niger, Aspergillus kanagwensis, Aspergillus nidulans limited at level two. This difference may be due to variable quantity of nutrients or aeration. This needs further work. Brandl et al. (2001) has reviewed the bacteria and fungi leaching the metals from mineral resources. The bacteria including ***Pseudomonas fluorescens*** and *Thiobacillus ferrooxidans* were detected by him. Microbiological analysis reveals that the numerous fungal as well as bacterial species were found such as Aspergillus niger, Aspergillus flavus, Penicillium sp., Aspergillus terreus, Aspergillus kanagwensis, Aspergillus nidulans and Trichoderma. Hamdy and Williams (2001) worked out a total of 150 cultures were isolated from treated bauxite residue and species detected were from bacteria like Pseudomonas, Bacillus, Lactobacillus, Leuconostoc

micrococcus, Staphylococcus, Flavobacterium and Enterobacter. Tyagi et al. (1994) isolated the fungal species like Aspergillus niger, Penicillium notatum, Penicillium simplicissimum and Trichoderma viridae from red mud and used for the exploitation of aluminum. Yang et al. (2006) showed diversified microbial community from copper mine which revealed the existence of bacterial species of Pseudomonas, Sphingomonas, Acidiphilium, Caulobacter, Candidatus, Azospirillum, and *Acidithiobacillus*. In addition they also confirmed the presence of more than 100 species belonging to fungi such as Apsidea, Alternaria, Aspergillus, Botrytis, Candida, Cephalosphoriuim, Chitomium, Cladosporium, Fusarium, Glomus, Monelia Mucor, Paicilomyces, Papularia Rhizoctonia, Rhizopus, Rhototrula, Sacchromyces, Sclerotium, Trichoderma and different unidentified fungi have also been recorded.

In addition the microbes occurred in the mines and industrial wastes were described by several authors (Table1 and 2). In the present investigation all these fungal species have been recorded. Most of the fungal species and the bacteria *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** were also isolated.

Microbial analysis of fly ash showed occurrence of Aspergillus niger, Aspergillus flavus, Penicillium sp., Aspergillus terreus, Fusarium, Aspergillus carboniferous and Helminthosporium were detected. The bacterial and actinomycetes population was also screened.

The metal extracting microbes, *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** were isolated from the bauxite ore. Morphology and biochemical characteristics of Pseudomonas and Thiobacillus isolates were studied. Both the bacteria were gram negative rods, and non motile in nature. Biochemically it was seen that none of the Pseudomonas isolates gave positive reaction for indole formation, nitrate reduction, Urease formation, gelantin hydrolysis and maltose utilization whereas Thiobacillus isolate showed oxidase, citrate utilization methyl red, indole production and Voges Proskuer test negative. On the contrary both the bacteria showed positive glucose fermentation, Catalase, and for Thiobacillus isolate Catalase, starch hydrolysis and growth on thiosulphate were found positive. This biochemical data in the present study support the findings of Simine and Gadd (1998). The morphological dimension was found 1- 4 µm long gram negative rods (Burgeys Manual of Systematic Bacteriology, 1998). Glucose fermentation of ***Pseudomonas fluorescens*** was also reported by Uzair et al. (2006) while fluorescence of Pseudomonas under UV exposure and growth on Kings medium have been worked out by Hossain et al. (2004). However, De-Siloniz et al. (1993) reported that there was no chemoorganotrophic growth of *Thiobacillus ferrooxidans* with glucose. The growth with glucose was also confirmed by Tuovinen and Nicholas (1977). For **Pseudomonas fluorescens**, fluorescence on Kings medium and growth at 10 to 45 ^{0}C were also confirmed by Prakash et al. (2007).

The effects of pH, NaCl concentration and temperature on the growth of and *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** isolates were studied. The pH 7.0 to 8.0 was highly favourable for the growth of ***Pseudomonas fluorescens*** and for *Thiobacillus ferrooxidans* pH 1.5 to 2.5 was highly suitable (Kantachote and Innuwat, 2004). An isolated strain grew at the pH range of 2.0 to 7.0 with an optimum pH of 6.5 but in our finding pH 2.0 was better for the growth of Thiobacillus isolates.

Hong-bo et al. (2007) studied the effect of temperature on *Acidithiobacillus* caldus grew at temperature varying from 15 ^{0}C to 60 ^{0}C. The yield of biomass was increased with increasing temperature (from 15 ^{0}C to 45 ^{0}C). The optimal growth temperature was 45 ^{0}C. Kantachote and Innuwat (2004) observed that all isolated strains of Thiobacillus grew at all temperatures from 25 ^{0}C to 45 ^{0}C but the optimal growth was between 30 to 35 ^{0}C.

Study of salt tolerance in metal extracting microbes is important for the bioextraction of the metals because the ore and fly ash contain metal salts. Hence effect of NaCl concentration on the growth of Thiobacillus and Pseudomonas isolates was studied and result showed that Thiobacillus and Pseudomonas could tolerate upto 4.5 %. Above 4.5% it was found inhibitory. Membray and Burlot (1994) investigated that the bacterial growth was inhibited by 5 % NaCl concentration. Nicodeme et al. (2008) analyzed the growth of Pseudomonas isolate was significantly slower in presence of 5 % NaCl concentration while Prakash et al. (2007) isolated and characterized Pseudomonas sp. which tolerate NaCl concentration upto 5 %.

Adapted strains of *Thiobacillus ferrooxidans* were more efficient in aluminum and iron extraction. **Pseudomonas fluorescens**, however, did not show any difference with the non-adapted one. Vachon et al. (1994) investigated that red mud adapted bacteria required 8 days for leaching while non-adapted bacteria required 15 days. The reason is that the more acid was produced by the adapted bacteria and hence more aluminum was solubilized from red mud i.e. 2350 mg of aluminum per liter (47 %). 730 mg of aluminum per litre (14 %) was estimated for non adapted bacteria. This finding was also confirmed by the work of Shahverdi et al. (2001). Bayat et al. (2008) have worked with adapted and non-adapted bacteria and it was observed that in the presence of non-adapted bacteria, the extraction of Zn and Fe was less than 6 % and 2 % after 21 days leaching respectively. In the presence of bacteria, rates for both Zn (25 %) and Fe (16 %) increased markedly. Kondali et al. (2004) in his findings proposed that the better yields in ferrous and nickel recoveries were obtained by adapted cultures (83.5 %) when compared to unadapted cultures; it may be due to increased metal tolerance level of adapted bacteria.

Along with the adaptation technique the induction of mutation is also effective. In the present investigation in order to increase the metal extracting efficiency of metal extracting microbes they were subjected to mutagenic treatment, such as UV irradiation and chemical mutagen, EMS. Out of 30 UV-mutants and 30 EMS mutants, two mutants from each mutagenic treatment of *Thiobacillus ferrooxidans* were efficient for extraction of aluminum and iron. EMS mutant-22 and UV mutant-26 were more efficient for extraction of both the metals whereas UV mutant -24 and EMS mutant-22 of ***Pseudomonas fluorescens*** also gave maximum aluminum and iron extraction. This work was supported in case of mutagenic breeding of silver resistant *Thiobacillus ferrooxidans* by Ling et al. (2007). The UV-irradiation was used for the induction of mutation for the period of 0, 30, 60, 90, 120 and 250 seconds at the power of 30 w, but in the present work irradiation time was increased upto 20 minutes. This confirms that *Thiobacillus ferrooxidans* have great resistance to the UV treatment and the dose of UV-irradiation was able to induce the mutation successfully. El-Bestawy et al. (1998) induced mutation through chemical and physical mutagenic agents resulted in mutants of Bacillus sphaericus, Bacillus licheniformis, Arthrobactor sp. and Staphylococcus aureus were superior over their wild types in removing heavy metals. During study it was observed that most of the bacterial mutants obtained by either UV-irradiation or ethidium bromide treatment showed higher removal efficiency for metals than their wild types. Xue-Ling et al. (2005) demonstrated that the mutated *Acidithiobacillus ferrooxidans* using microwave and UV irradiation indicated that the activities of the mutated strain was increased by 39.96 % and the leaching rate was increased by 31.44 % compared with that of the wild type progeny.

Different modified media for autotrophic microorganisms have been developed and studied by different researchers. Hence in present investigation different media have been used to extract metal bauxite ore and fly ash. 9k medium without glucose was found better in metal extraction. Mokita et al. (2004) also observed that 9k medium was effective source of nitrogen and phosphorus which enhance the growth of organisms. Nemati and Webb (1997) reported maximum iron oxidation and metal bioextraction with 9k medium. Ghosh et al. (2004) investigated that 95 % Co and 100 % zinc could be extracted with 9k medium while other media

are less effective. Same type of results was obtained by Zamani et al. (2005) and Drougui et al. (2005).

The pH is an important parameter as it influences, not only bacterial growth but also controls some important physico-chemical interaction in the system. In the present investigation, effect of pH on extraction of metals from bauxite ore was studied. At the pH 2.0, *Thiobacillus ferrooxidans* gave higher extraction of metals. This result was in agreement with the result of Karavaiko (1980) who argued that the bioleaching processes are usually carried out in acid environment, preferably at pH 1.7-2.4 when metal ions remain in solution. Important feature of *Thiobacillus ferrooxidans* showed its ability to tolerate high acidity as well as high concentration of metal ions. This characteristic feature was exploited in biomining to extract metals from their concentrates (Cawlina et al., 1990). Bharathi et al. (2008) also achieved 82 % nickel extraction from copper floatation concentrate at pH 2.3. However, Kodali et al. (2004) noted that the operating conditions, such as solid's concentration, agitation, aeration, and nutrient requirement residence time and pH regulation were optimized for selective leaching of nickel. They studied that the pH plays a critical role for the maintenance of cyclic process of $Fe^{2+} \leftrightarrow Fe^{3+}$ and eliminate the ferric ion precipitation which controls the whole leaching process. With the onset of pH stabilization, at an optimum 2.3, *Thiobacillus ferrooxidans* cells entered the exponential phase of growth and were metabolically highly active during this period, leading to further generation of biologically synthesized sulfuric acid. It was documented (Chan and Ting 1995) that the increase in the pH leads to the increasing negative charges on the bacterial cell surface with the subsequent attraction with positively charged metal ions. Bharathi et al. (2008) emphasized that the fastest leaching rates are achieved under oxidized conditions and at low pH values, indicating bacterial activity is responsible principally for the oxidative leaching of sulphide minerals.

At alkaline pH ***Pseudomonas fluorescens*** gave better results when both the aluminum and iron extraction were considered. The pH of the reaction medium is a vital factor in metal solubilization so optimization of these factors is essential.

In most cases metal solubilization is the result of organic acid production although other mechanism such as production of bacterial metabolites and sidrophores have also attributed to solubilization. Similar results were obtained by Nautiyal et al. (2000) and Nahas (1996). Kim et al. (2003) reported that metal solubilization was associated with sharp decline in pH along with other factors.

In the present investigation effect of temperature on the extraction of metal by both the bacteria was also studied. Temperature 36 ^{0}C and 32 ^{0}C were found most suitable for the extraction of both the metals aluminum and iron by *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** respectively. Bharathi et al. (2008) have extracted 82 % nickel from copper floatation concentrate at 30 ^{0}C temperature using same bacteria hence this supports the result.Mishra et al. (2008) have also studied the recovery of metals at three different temperatures 25 ^{0}C, 32 ^{0}C and 40 ^{0}C during leaching experiment Nickel and Vanadium released to solution did not show any significant change due to the said range of temperature, however, molybdenum dissolution into solution was decreased with increased temperature. Hossain et al. (2004) also studied the effect of temperature on bioleaching of Zn (II) where they stated that optimum temperature is 35 ^{0}C at which the maximum Zn (II) biooxidation was found. Ollubambi et al. (2008) found that the intensity of bacterial leaching for both Zn and sulphide (minerals) was dependents on the rate of supply and dissolution of O_2 and CO_2. The dissolution of O_2 and CO_2 decreased with increase in temperature.

Hossain and Ananthraman (2005) during experiment noticed that the mesophiles grew best within the temperature range at 20 ^{0}C to 50 ^{0}C. Every type of microbes has an optimum,

minimum and maximum growth temperature. Duarte et al. (2006) summarized that the temperature directly affects the microorganism behavior, and it was observed that small variation (± 5 ^{0}C) in the operating temperature could lead to significantly affected copper extraction rate. Chan and Ting (1995) noted that increased temperature increases the metabolic activity of bacteria which resulted in the increasing metal extraction capacity.

Lorenzo et al. (1997) studied the effect of temperature on bioleaching of copper from chalcopyrite by the strains of *Thiobacillus ferrooxidans* and put forward that the *Thiobacillus ferrooxidans* culture at 38 ^{0}C reached copper dissolution values higher than all the other three cultures. The strain TM4 adapted at 45 ^{0}C displayed a copper bioleaching activity similar or even slightly higher.

When ***Pseudomonas fluorescens*** was considered, at 32 ^{0}C temperature gave better results for the extraction of aluminum and iron while results obtained by Shahab and Ahmed (2008) showed that 30 ^{0}C is the more suitable temperature for metal solubilization. At the temperature less than 30 ^{0}C, the metal solubilization was weak. However, solubilization at extreme temperature has also been reported by few workers such as Nautiyal et al. (2000) and Nahas et al. (1996) who had reported the solubilization at 45 ^{0}C by a desert soil isolates. Johri et al. (1999) have reported solubilization at 10 ^{0}C which clearly suggest that bacteria adapt to their indigenous environment therefore their metabolic activities are linked to the temperature of the environment.

Incubation period indicated that metal extraction increased with the increase of incubation period by both the bacteria. Similar results were worked out by Cawlina et al. (2000) the author indicated that metal bioleaching was increased with the incubation time. Hossain et al. (2004) in his study showed that the maximum Zn (II) bioleaching was 68.46 % after 192 hours.Duarte et al. (2006) also studied the effect of residence time on bioleaching of copper from chalcopyrite and found that percent copper extraction increased with increased residence time of 8 days (73 %) and for 14 days (83 %). Moreover, Rezza et al. (1997) investigated that as per the length of incubation metal extraction also increased upto 95%. In addition some others also agreed as per the above result (Sand et al., 2001).

In the present investigation magnitude of substrate concentration was also studied and it was observed that 5-6 gm of bauxite ore concentration was found to be better for aluminum and iron extraction by both the bacteria. According to Ballester et al. (1989) the percentage of copper bioleaching increased when the pulp density decreased. It is because of the adaptation period of bacteria, as the pulp density increase adaptation to that density required a long period due to that metal bioleaching decreased. These results can be explained by the fact that as the solid liquid ratio decreases the conditions of the hydrodynamics of the fluid involved in the process improve and thus the metal leaching is enhanced. This is because the diffusion of the O_2 and Co_2 has limiting effect on the development of the microorganisms. Due to more solid material in the medium the availability of oxygen to the bacterium decreased hence the process become attenuated. Brandl et al. (2008) also postulated that the pulp density has a major influence on bacterial growth and activity, whereas increased pulp density more than 50 g/l significantly affect the metabolic activity of bacteria. Cawlina et al. (2000) in his experiment observed that the concentration of the leached metals were considerably higher in the leaching solutions with higher pulp densities, although further increase in the pulp densities caused decrease in the yields of bioleached metals.

Pradhan et al. (2008) have studied different pulp concentration and also agreed that the increasing pulp density decrease the metal extraction capacity due to poor diffusion of oxygen in the medium (as the strain used as an aerobic bacteria). The decrease in leaching beyond certain limit can be attributed to improper contact of the medium with the ore particles. Vachon

et al. (1994) studied different pulp concentration on bioleaching of red mud sludge by *Thiobacillus ferrooxidans* and revealed that 10 % and 15 % of pulp concentration inhibit the bacterial growth.

Woznick and Huang (1991) have indicated that the efficiency of metal dissolution is strongly influenced by the solid concentration of the leach slurry. Bayat et al. (2008) investigated that the increase in pulp density results in a decrease in percent extraction of both Zn and Fe. It might be due to increasing pulp density which created a condition with higher toxicity and shear stress and decreased the mass transferred that could result in the decreased percent extraction of Zn. Duarte et al. (2006) showed that pulp density may affect not only the hydrodynamics regime and reactor design but it also affects the microorganism's behavior. Hence the appropriate pulp density is required for enhancing metal extraction.

Present investigation reveals the effect of agitation speed on bioextraction of metals. It was found that aluminum extraction was increased with increase of agitation speed. Extraction of iron by *Thiobacillus ferrooxidans* gave same results. In case of ***Pseudomonas fluorescens*** increase of agitation speed increased aluminum and iron extraction. These findings also confirmed as per the work of Kodali et al. (2004) which suggest that 140 rpm agitation speed gave 83 % of nickel extraction. Moreover Hossain et al. (2004) also studied the effect of shaking speed (rpm) and found that the optimum shaking speed is 160 rpm at which maximum bioleaching of Zn is 74.85 % and 71.50 % respectively. They indicated that secretion of enzymes responsible for bioleaching is highest at the same shaking speed with maximum bioleaching of zinc sulphide ore. Somasundram et al. (2000) demonstrated that the speed of rotation from adequate point in conditioning stage the bacteria cannot depress pyrite and recovery of copper were increased since it destroys bacteria's walls and increases shear stress in increasing speed of rotation.

Particles of different sizes have different surface area, and hence the efficiency of bacteria attachment and metal dissolution depend on the bauxite ore particle size. In the present investigation magnitude of particle size on the bioextraction of metals from bauxite ore by both the organisms was studied. It was noted that bioextraction was decreased due to the increase in the particle size. To optimize these parameters low grade copper sulphide ore was tried by Kolahdoozun et al. (2004) and showed that the degree of bacteria attachment to the particles of ore increase. It was seen that the bioleaching process was the most effective when the ore particles sizes were in range of 90-125 nm. These results were also advocated by Cawlina et al. (2000). According to Ballester et al. (1989) degree to which the particles are ground has a strong influence on the microbial oxidation of the mattes. It is evident that smaller the particle size of the solid the greater the proportion of copper leached but after certain level decrease in size do not affect bioleaching. Pradhan et al. (2008) investigated that after 55 days of leaching it was observed that at finest fraction that is 200-250 mesh metal dissolution was enhanced. Due to finer fractions the bacteria as well as the ferric ion are exposed to more surface area and therefore the leaching rate is enhanced.

Effect of inoculum concentration of *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** was studied. It was noted that increase in inoculum concentration increases aluminum and iron contents. These findings were also confirmed by the work of Kodali et al. (2004) who reported that the increase in inoculum concentration also increase nickel extractions from the copper floatation concentrate. Wang et al. (2008) explained the results obtained from the bioleaching experiments indicated that the efficiency of copper extraction depends on all of the mentioned variables especially pulp density and the inoculation cell numbers. As the inoculation cell number increased copper dissolution reached to 51.34 % for chalcopyrite while it was 72.35 % for the bornite after 30 days.

The presence of some anions and cations at certain levels in the bioleaching environment may exert an inhibitory effect on the growth and hence this directly affects the leaching activity of bacterial culture. Addition of NaCl in the flask resulted that the aluminum and iron extraction reduced with the increase of NaCl concentration. Deveci (2002) investigated that the salinity upto 8 % affects the bioleaching activity of mesophilic as well as moderately and extremely thermophilic strains of bacteria during the bioleaching of a complex Zn/ Pb sulphide ore. Kozuki et al. (1995) reported during bacterial biodegradation of organic contaminants in Tridal river revealed that salinity content affect bacterial respiration and population. Kozuki and workers observe that the greater the change in salinity the greater the effect on the bacteria. With stable level of salinity increased the surviving level of bacteria and the resulted in population increase.

Weston et al. (1994) argued that the deleterious effect of salt was not due to chloride present, but rather due to the coexisting ions such as sodium leading to the formation of Jarosite precipitates. Dew et al. (1997) suggested that high concentration of chloride in solution could damage the membrane of the bacteria. From an industrial point of view it might be of importance to note that Pseudomonas plecoglossicida is able to grow in NaCl solution of upto 50 g/l. This finding was supported by Brandl et al. (2008).

Due to addition of NaCl in the flask resulted that the aluminum and iron extraction decreased which attributed to work of Rosado et al. (1998) who reported that this might be due to reasons, either two stresses (metal and sodium chloride ions) at the same time may harm cell growth and proliferation which results in less efficiency of solubilization or it might be possible that too much chloride ions may chelates or neutralize proton ions or acid produced in reaction media.

In the present investigation the metal extraction by cell free extract (metabolites) of *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** was studied. It was seen that aluminum and iron extraction was highly increased in presence of complete cells rather than the cell free extracts. The cell themselves might be responsible for the accumulation of metals by acting as nucleus or this may be because of surface charges present on the cell wall. The mechanism of bioleaching are usually described in terms of either direct bacterial attack on metal sulfides or an indirect attack via ferric iron (Tyagi et al., 1990; Fowler and Crundwell, 1999) enhanced the solubilization of Cadmium and Nickel from contaminated sediments. Rahman and Gul. (2003) investigated that the indirect leaching method was slow for the solubilization of copper from the ore and only 28.52 % of copper leached in 70 days while on the other hand 87.57 % with direct leaching. Moreover Mishra et al. (2008) observed that in the presence of cell plus metabolites recovery of Nickel, Vanadium and Molybdenum were found to be 88.2 %, 93.0 % and 17.4 % respectively within 7 days incubation period. The result obtained by Jekic et al. (2007) suggested that metal leaching efficiency increased when suspension with *Acidithiobacillus ferrooxidans* was used, as compared to in absence of complete cells,

In this work when ***Pseudomonas fluorescens*** and *Thiobacillus ferrooxidans* were combined with other bacteria like **Pseudomonas fluorescens**, Pseudomonas aeruginosa, Bacillus cereus, Bacillus megaterium aluminum and iron extraction was decreased by the addition of all these microbes. These results supported the finding of Blais et al. (1992) they showed that only 40 % Ni solubilization from municipal sludge with an iron oxidizing and heterotrophic mixed cultured during 10 days of microbial leaching.

Some researchers have studied bioleaching using pure cultures and mixed cultures, and compared their efficiency in terms of metal dissolution (Battaglia et al., 1994). However it is very complex to explain how consortia of bacteria appear to work, compared to pure culture. Thus far, no one has proposed any bioleaching model of mixed cultures. Cheng and Hu (2007) reported that copper and iron extraction increased 53 % and 44 % with mix microbial consortia.

Pseudomonas fluorescens gave higher extraction of both the elements when used in combination with other bacterium like Pseudomonas aeruginosa Gopalan et al. (1993) noted that the iron removal was increased with mixed culture of iron resistant microbes as compare to individual. El-Bestaway et al. (1998) postulated in his experiment that the mixed wild types culture improve the removal efficiency for Fe, Ni and Pb by 1.8, 3.1 and 2.5 fold respectively when compared with the removal efficiency achieved by the individual wild type species.

Both of these gram negative bacteria gave better results when used for extraction of aluminum and iron these confirmed with the observation made by Beveridge et al. (1981) which explains that in gram negative bacteria, lipopolysacchride (LPS) layer can be highly anionic and extends beyond the outer membrane proteins; this layer has been implicated as the major source of metal binding in this group of bacteria.

Synergistic effect of *Thiobacillus ferrooxidans* and Aspergillus niger for the extraction of aluminum and iron showed the decrease in aluminum and iron extraction. Some bacteria and fungi can promote selective and non selective leaching of one or more metal constituents from an ore or other industrial waste material (Ehrlich, 1996). The acids may be organic or inorganic. Groudev and Groudev (1986) were able to leach aluminum from clays with oxalic and citric acids formed by Aspergillus niger. Alibhai et al. (1993) were able to leach nickel selectively from low grade Greek laterites with citric acid produced by various species of Aspergillus and Penicillium with organic acids, (such as acetic acid or lactic acid) where as organic acids are harmful to acidophiles because they function as un-couplers of the respiratory chain at low pH by diffusion of the protonated form into the cell followed by dissociation of a proton (Lavelle et al., 2005).

Castro et al. (2000) investigated the role of bacteria from the genera Bacillus and Pseudomonas and fungi from the genera Aspergillus and Penicillium in leaching of zinc and nickel from two different silicates and found that microbes in combination solubilized metal efficiently as compare to alone. Tzeferis et al. (1991) also demonstrated the fungal efficiency in metal leaching from the low grade nickel oxide ores to leaching by Aspergillus sp. and Penicillium sp. while nickel recoveries as high as 90 % have been observed (Sukla and Panchanadikar, 1993). Tzeferis et al. (1994) leached upto 60 % and 50 % of Ni and Co respectively from non-sulfidic nickel ores using Aspergillus sp. and Penicillium sp. They showed the presence of citric, oxalic and organic acids in leach liquors, indicating their role in the bioleaching process.

The amount of mineral ore reserve is limited and they may be exploited day by day at alarming rate so they may be exhausted unless they are conserved. To overcome this problem dependence on ore reserves minimized. Thorough utilization of different industrial as well as municipal wastes and low grade ore now being considered as sources of metals. Bioleaching applied with chemolithotrophic and heterotrophic microorganisms is an important biological process for industrial and municipal waste in order to recover valuable metals and detoxify them. Especially, low prices of raw material waste increase this importance much more. Bioleaching is a promising technology for recovering valuable metals e.g. Fe, Zn, Ni, Co, Cr and Cu, Al from waste material. These metals can be recovered and recycled by metal processing industries. The wastes contaminated with valuable metals may consider being secondary resources. Efforts have been made to go for bioleaching of the valuable metals from fly ash. It may afford a viable and economical process in future within appropriate economic constraints.

In the present investigation efforts have been made to detect the magnitude of adapted and non adapted strains on bioextraction of metals from fly ash with the help of *Thiobacillus ferrooxidans* and **Pseudomonas fluorescens**. It was noted that aluminum, iron and manganese extraction was increased with adapted strain than that of non adapted strains during experiment on adapted and non-adapted Thiobacillus. It was supported by the observations by Vachon et al.

(1994) who found that more acid (H_2SO_4) was produced by the adapted bacteria and thus more aluminum was solubilized from red mud, 2350 mg/l (47 %) for the adapted and 730 mg of Al/l (14 %) for non-adapted bacteria. The adapted Pseudomonas species was investigated for the recovery of metals from the coal pile run off. The analysis of elemental composition of metals precipitate recovered from the bacterial biomass by transmission electron microscopy and energy dispersive x-ray analysis revealed the presence of metals originally present in the wastewater (Ibeanusi et al. 2003).

Using fly ash the extraction of metals by *Thiobacillus ferrooxidans* was studied. It was found that the pH 1.5 to 2.5 was favorable for the extraction of elements. However, ***Pseudomonas fluorescens*** showed aluminum, iron and manganese extraction upto pH 8.0. Furthermore iron extraction was also increased from pH 5.0 to 8.0. The pH is critical criterion for the extraction of metals because metals dissolved in acids (at low pH). The effect of pH on the metal extraction from fly ash in bioleaching process was worked out by Pathak et al. (2008). They estimated the solublization of Cu, Ni, Zn and Cr at different initial pH. Wang et al. (2002) worked out with the pH requirement for isolated indigenous *Thiobacillus ferrooxidans* for bioleaching of heavy metals from wastewater sludge in Hong Kong and showed that the removal of Zn, Cu, Ni and Cr was done efficiently at high initial pH which is 5 to 7.0. This was found controversial to the present results. This may be due to other mechanisms induced by *Thiobacillus ferrooxidans*.

Pseudomonas fluorescens was found better for the bioextraction of aluminum and iron at the pH ranged 7.0 to 8.0 (almost neutral) it might be due to Pseudomonas growth at this pH range. The higher pH values (In presence of fly ash) stimulate oxalic and gluconic acid production. Similar results were obtained by Grewal et al. (1995). Dronawat et al. (1995) however, the growth was found accompanied by an increase in pH. The extensive increase in pH for the experiments with ***Pseudomonas fluorescens*** may also attributed to production of payoverdine molecules since the payoverdine legend has the ability to take up four protons. Its release to the solution could not result in a promoted increase in pH. Leung et al. (2000) explained the significant role of pH in bioextraction. At lower pH sorption of hydrogen ions was preferred over that of metal ions, but at higher pH, more metal ions would be taken up.

One of the main issues in metal extraction from wastes materials is the concentration of the waste amount in the reaction media. Because it was reported that non diluted waste material had an inhibitory effect on bioleaching organism (Picher et al., 2002; Solisio et al., 2002; Isligaki et al., 2005). The effect of initial substrate concentration of waste material was studied and it was observed that, in general 4 to 6 g fly ash gave more extraction of Al, Mn and Fe by both the organisms. This is confirmed by the work of Bosecker (1997) who reported the effect of substrate concentration (fly ash) indicated that the extraction of aluminum and iron by *Thiobacillus ferrooxidans* increased with the increase in substrate concentration but beyond optimal (4-6 gm) it was found decreased. This might be due to saturation effect of the enzyme (Mokita et al., 2004).

Metal extraction was increased with the increase of incubation period for both the organisms. These results are in agreement with other studies reported in the literature. Sathiya and Ting (2005) demonstrated that after 60 days of incubation period the highest metal extraction values recorded were 54.5 % Al, 58.2 Ni and 82.3 % Mo. In another study similar organism was used which is *Thiobacillus ferrooxidans*. Obtained results were 9 % Ni, 23 % Fe, 30 % Al, 36 % V and 64 % Sb were transformed into the soluble forms after mobilization (Aung and Ting, 2005). However, Brombaacher et al. (1998) worked out semi continuous biohydrometallurgical processing of fly ash from municipal waste incineration was performed in a laboratory scale leaching plant (LSLP) and it was found that leaching efficiency obtained for metals zinc were upto 81 % and leaching efficiency for Al were upto 52 % whereas highly toxic Cd was

completely solubilized within 30 days of incubation period. Moreover Lombardi et al. (2006) investigated that the bioleaching of Mn, Al, Zn, Cu and Ti ions in municipal sewage sludge were dependent on residence time. As residence time increase metal solubilization also increases these confirms with other results also. Bormbacher et al. (1998) pointed out that the leaching is obtained for economically most interesting metal zinc was 99 %. Leaching efficiency for Ni, Cu and Cr were 96, 81 and 71 % within 100 hours incubation period.

Bioextraction of aluminum, manganese and iron from fly ash was studied at different temperature. It was found that there was increase in aluminum, mangnese and iron with the increase of temperature upto 38 ^{0}C whereas in case of ***Pseudomonas fluorescens*** metal extraction was increased upto 34 ^{0}C.Many authors' investigation shows that the efficiency of bioleaching is depend on temperature (Chen and Lin, 2000) with its optimal, at 37 ^{0}C however, the optimum incubation temperature ranging from 30 to 37 ^{0}C suitable for metal leaching from the industrial wastes, considering the increasing cost with increase in temperature (Gomez et al., 2000; Lee et al., 2001).

Increasing temperature in the range of 20 to 35 ^{0}C was found to be enhanced the biological oxidation rate of ferrous ions by *Thiobacillus ferrooxidans* (Nemati and Webb, 1997). Deng, (2002) also studied the temperature effect in the range of 20 to 45 ^{0}C. It was observed that the biooxidation range of iron and arsenic using *Thiobacillus ferrooxidans* was highest in the range of 28 to 32 ^{0}C which leads to production of ferric iron as well as H_2SO_4; hence dissolution of metal is increased.

The metal extracting bacteria were analyzed at genetic level. To locate the genes the plasmid curing techniques was used due to which it was confirmed that the genes responsible for the metal extraction are not plasmid borne but these may be present on the genomic DNA. This is very important in breeding point of view when the horizontal gene transfer among bacteria is studied. The results were in confirmation with Ade (2003), who confirmed the genes, for antagonistic property in Pseudomonas, were located on plasmids by the same technique of curing.

To differentiate the genetic profile of *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** and their respective mutants the ISSR markers were used. Ziethkiewicz et al. (1994) reported this technique for the first time, where microsatellites anchored at the 3$^{'}$ end are used for amplifying genomic DNA. The anchor also allows only a subset of the targeted inter-repeat regions to be amplified, thereby reducing the high number of PCR products expected from the primers of inter-repeat region.

ISSR marker was found simple and capable of producing reproducible and greater polymorphism than other marker system (Wolfe et al. 1998). The efficiency of ISSR markers than RAPD markers is higher, which is well visualized from higher frequency bands and polymorphic information content values (Chattopadhyay et al., 2005).

To differentiate the mutants from the wild strains of *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** the ISSR markers was used which showed polymorphism at remarkable level. The construction of dendrogram showing relationship between Pseudomonas and Thiobacillus was possible. There were three clusters showing the similarity indices with their respective mutants. *Thiobacillus ferrooxidans* showed value of 0.24138 with the mutant UV-26. The ***Pseudomonas fluorescens*** was similar by the value of 0.25926 with its mutant UV-24. Similar work was carried out by Hussein et al. (2008) who used the same marker for molecular identification and genetic relationships of strawberry verities (Sokal and Michener, 1985; Zietkiewicz, et al. 1994).

Proteomic technologies are powerful tools for examining alterations in protein profile. To investigate the a possible correlation between metal ion tolerance and protein profile of the *Thiobacillus ferrooxidans* mutant UV-26 and ***Pseudomonas fluorescens*** mutant UV-24 showed similar types of protein profile pattern when compared in absence of respective metal. Similar results were found by Shilev et al. (2007) for Thiobacillus and Pseudomonas. On the basis of above result it was noticed that in presence of metals these bacteria produces a minor types of proteins. For critical analysis further work is needed.

The FTIR analysis was done in the present investigation in order to determine the type of bonding between the metal extracting microbes and their respective metal sources. This is a promising technique in which the samples can be characterized as well as reaction products can be identified at various temperatures with the help of Fourier Transform Infrared (FTIR) Spectroscopy.

An Infra Red spectrum of a given compound is unique and can therefore serve as a fingerprint for the compound. *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** mutants treated with bauxite and fly ash along with untreated one were analyzed using this technique. Bonding of O-H as well as the functional groups, CH_3 and COO^- were observed. The work carried out done the surface chemical studies of *Thiobacillus ferrooxidans* in presence of different metal concentration also support the result. Delvasto et al. (2009) who reveal the chemical nature of the exopolymeric material surrounding a cell and also other soluble exopolymers present in the liquid media.

The perusals of above discussion indicate that optimal parameters can be used for the extraction of aluminum and iron from bauxite ore and fly ash from thermal power station.

SUMMARY AND CONCLUSION

1] Physiochemical analysis of bauxite ore was carried out. There was difference in water holding capacity in the bauxite ore. Total carbon, total nitrogen, phosphorus, potassium, sodium were also analyzed. The nitrogen and carbon content were twice and six times more in the bauxite as compared to fly ash. The pH of the fly ash was slightly alkaline.

2] EDX-analysis was done by X-ray crystallography of bauxite ore surface; aluminum silicon, titanium, and iron were detected. Aluminum was found at 46.27 mass percent and iron 46.87 mass percent, whereas other elements were found less in quantity. The fly ash show 20.20 mass percent of aluminum and 25.04 mass percent of silicon. Other elements like iron 4.24 and magnesium 1.01 and manganese 0.64 mass percent.

3] XRD analysis of bauxite ore and fly ash was carried out in order to find out the mineralogical phases. The phases such as diospora, kaolinite, gibbsite, illite2M#2, hematite, goethite, bohemite, trydymite-2OHsyn, biotite, albite disordered, calcite, wallastonite and anatase were reported in bauxite while in fly ash quartz, coesite, sodium aluminum silicate, tricalcium manganese orhosilicate, magnesium silicate, calcium aluminum silicate, ferric oxide, mullite, magnetite and hematite were found.

3] Microbial analysis at different layers of bauxite ore indicated the microbes occurred at different depths. Aspergillus flavus, Aspergillus fumigatus, Fusarium oxysporum, Rhizopus continued to be present at all levels; whereas Aspergillus niger, found at 4th level. Aspergillus kanagwensi and, Aspergillus nidulans, were limited at level 2, however, Trichoderma sp. Aspergillus terreus were absent at level 1. All the fungi were found decreased with the increase in the depth quantitatively.

4] Microbial analysis of fly ash showed occurrence of the fungal species like Aspergillus niger, Aspergillus flavus, Penicillium sp., Aspergillus terreus and Fusarium, Aspergillus carboniferous and Helminthosporium. Bacteria and actinomycetes population were also analyzed.

5] Among the microbes ***Pseudomonas fluorescens*** and *Thiobacillus ferrooxidans* were isolated from the bauxite ore. They were identified with the help of Bergey's Manual of Systematic Bacteriology by conducting Gram staining, colony characteristics and biochemical tests.

6] The effect of pH, NaCl and temperature on the growth of ***Pseudomonas fluorescens*** and *Thiobacillus ferrooxidans* isolates were studied. The pH 7.0 to 8.0 was found highly favourable for growth of ***Pseudomonas fluorescens*** while for *Thiobacillus ferrooxidans* pH 1.5 to 2.5 was highly suitable. Both the organisms could tolerate NaCl upto 4.5 percent. Effect of temperature was also studied. The maximum growth of ***Pseudomonas fluorescens*** was occurred at 30^0C and 35^0C whereas for *Thiobacillus ferrooxidans* 30^0C to 40^0C was found most suitable.

7] Both the bacteria, *Thiobacillus ferrooxidans* as well as ***Pseudomonas fluorescens*** were highly resistant to titanium, silicon, aluminum and iron whereas *Thiobacillus ferrooxidans* was resistant to all metal concentrations from 1mg/ml to 5mg/ml and ***Pseudomonas fluorescens*** tolerated upto 3mg/ml and above it showed decline in growth.

8] Adapted strains of *Thiobacillus ferrooxidans* were more efficient in aluminum and iron extraction. ***Pseudomonas fluorescens*** however, did not show remarkable difference.

9] Mutation of *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** was done by the treatment of ultraviolet irradiation and chemical mutagen, EMS and they were used for further studies. Out of 30 UV isolates of *Thiobacillus ferrooxidans* Isolate no UV-26 was efficient for extraction of aluminum and iron. EMS mutants of *Thiobacillus ferrooxidans* 22 and 26 were more efficient for extraction of both the elements, whereas UV and EMS mutants (UV-24 and EMS-9) of ***Pseudomonas fluorescens*** also gave maximum aluminum and iron extraction.

10] Different media like, basal salt medium, Marsh and Norris medium, 9K with glucose, T and K medium and 9K without glucose were used for the extraction of metals. Out of these the medium, 9k medium without glucose gave more extraction of metals in case of *Thiobacillus ferrooxidans*.

11] The effect of pH on the bioextraction of metals was also studied. The pH 2.0 gave higher extraction of aluminum and iron by *Thiobacillus ferrooxidans* whereas the pH 8.0 was found most suitable for extraction of aluminum and iron by **Pseudomonas fluorescens**.

12] Effect of temperature on the extraction of metals by both the bacteria was also studied. Temperature 36^0C and 32^0C was most suitable for extraction of aluminum and iron by *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** respectively.

13] The metal extraction was increased with the increase in incubation period by both the bacteria.

14] Five percent of bauxite ore concentration was found better for aluminum extraction by *Thiobacillus ferrooxidans* whereas iron extraction was maximum at 4% ore. In case of **Pseudomonas fluorescens**, aluminum extraction was higher at 4 gm bauxite ore concentration.

15] Aluminum extraction was increased with increasing agitation speed. Extraction of iron gave same results by *Thiobacillus ferrooxidans*. In case of ***Pseudomonas fluorescens*** also same pattern was observed.

16] Effect of particle size on the bioextraction of metals from bauxite ore by both the organisms was studied. It was noted that bioextraction was reduced due to the increase in the particle size. *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** gave maximum aluminum and iron extraction with smaller particle size.

17] Effect of inoculum concentration of *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** was studied. It was noted that increase of Inoculum concentration increased aluminum and iron contents.

18] The concentration of NaCl and extraction of metals by *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** was found inversely proportional due to inhibitory activity of NaCl.

19] The extraction of metals by *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** cells and cell free extract was studied comparatively and it was found that the extraction by cells was more rather than the cell free extract.

20] When ***Pseudomonas fluorescens*** and *Thiobacillus ferrooxidans* were combined with other bacteria like, Pseudomonas aeruginosa, Bacillus cereus, and Bacillus megaterium aluminum and iron extraction was decreased, while ***Pseudomonas fluorescens*** gave higher extraction of both the metals when used in combination with Pseudomonas aeruginosa only.

21] Synergistic effect of *Thiobacillus ferrooxidans* and Aspergillus niger, Aspergillus flavus, Penicillium and Rhizopus species was studied for the extraction of aluminum and iron. Addition of fungi in combination with *Thiobacillus ferrooxidans* decrease aluminum and iron extraction. More or less same types of results were obtained in case of **Pseudomonas fluorescens**.

22] Metal extraction from fly ash with the help of *Thiobacillus ferrooxidans* ***Pseudomonas fluorescens*** was also studied. The strains were adapted by sub culturing four times. The adapted strain and non adapted strain were treated with fly ash. It was noted that aluminum, iron and manganese extraction was increased with adapted strain than that of non adapted one.

23] Effect of pH on bioextraction of metals from fly ash by *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** and was also studied. The pH 2.0 to 2.5 was favourable for the

extraction of metal in case of *Thiobacillus ferrooxidans* while ***Pseudomonas fluorescens*** showed aluminum, iron and manganese extraction upto pH 8.0.

24] Bioextraction of aluminum, iron and manganese from fly ash was studied at different temperature. There was increase in aluminum, iron and manganese with the increase of temperature upto 38^0C in case of *Thiobacillus ferrooxidans* whereas in case of ***Pseudomonas fluorescens*** increased metal extraction upto 34^0C.

25] Metal extraction were increased with the increased of incubation period of both the organisms inoculated in fly ash.

26] The fly ash concentration reported for maximum extraction of aluminum, iron and manganese by both the bacteria was found to be 2 g/ 100 ml (w/v).

27] The location of metal extracting genes was confirmed by curing. The bacteria were treated with acridine orange and ethidium bromide for inactivation of plasmids. The acridine orange and ethidium bromide treated and non treated cells did not show any significant difference in bioextraction of metals indicating that the genes are not present on plasmids. The same was observed in **Pseudomonas fluorescens**. These must be located on genomic DNA.

28] The genetic diversity of metal extracting bacteria was studied by ISSR marker assisted profiling of genomic DNA. The wild strains of *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** have shown similarity index 0.25926. The similarity indices of the wild strains with their respective mutants were also determined. The similarity index of wild and mutant *Thiobacillus ferrooxidans* was found 0.24138. The similarity index of wild ***Pseudomonas fluorescens*** and its mutant was reported as 0.25926.

29] In order to study the protein profile at the time of metal extraction in the mutants of *Thiobacillus ferrooxidans* and **Pseudomonas fluorescens**, SDS-PAGE was used which revealed that the proteins separated by this technique showed almost similar bands. This needs further research.

30] FTIR analysis showed different bonding interactions of *Thiobacillus ferrooxidans* and ***Pseudomonas fluorescens*** mutants with the substrate, bauxite ore and flyash.

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[* Original not seen.]

ABBREVIATIONS USED

AAS	:	Atomic absorption spectrophotometer
AFLP	:	Amplified fragment length polymorphism
bp	:	Base pair (s)
CAS	:	Chrome azurol s.
CBBR	:	Coomassie brilliant blue R-250
CFA	:	Coal flyash
c.f.u.	:	Colony forming unit
CTAB	:	Cetyl trymethyl ammonium bromide
DES	:	Diethyl sulfate
DGGE	:	Denaturing gradient gel electrophoresis.
dNTPs	:	2'-deoxynucleoside 5'- triphosphate
EDAX	:	Energy dispersive x-ray analysis
EDTA	:	Ethylene diamine tetraacetate
EMS	:	Ethylmethane sulphonate
EPS	:	Exopolysaccharides
FTIR	:	Fourier transform infrared spectroscopy
IR	:	Infra red
ISSR	:	Inter simple sequence repeat
K Da	:	Kilo dalton
kb	:	kilo base pair (s)
LSC	:	Large spreading colony
MIC	:	Minimum inhibitory Concentration.
PAGE	:	Polyacrylamide gel electrophoresis
PCR	:	Polymerase chain reaction
PFGE	:	Pulsed field gel electrophoresis.
RAPD	:	Random amplified polymorphic DNA
SDS	:	Sodium dodecyl sulphate
SEM	:	Scanning electron microscope
TAE	:	Tris- acetate-EDTA
TBE	:	Tris-borate-EDTA
TCA	:	Trichloro acetic acid
TEM	:	Transmission electron microscope
TEMED	:	N,N,N,N-tetramethyl ethylene diamine

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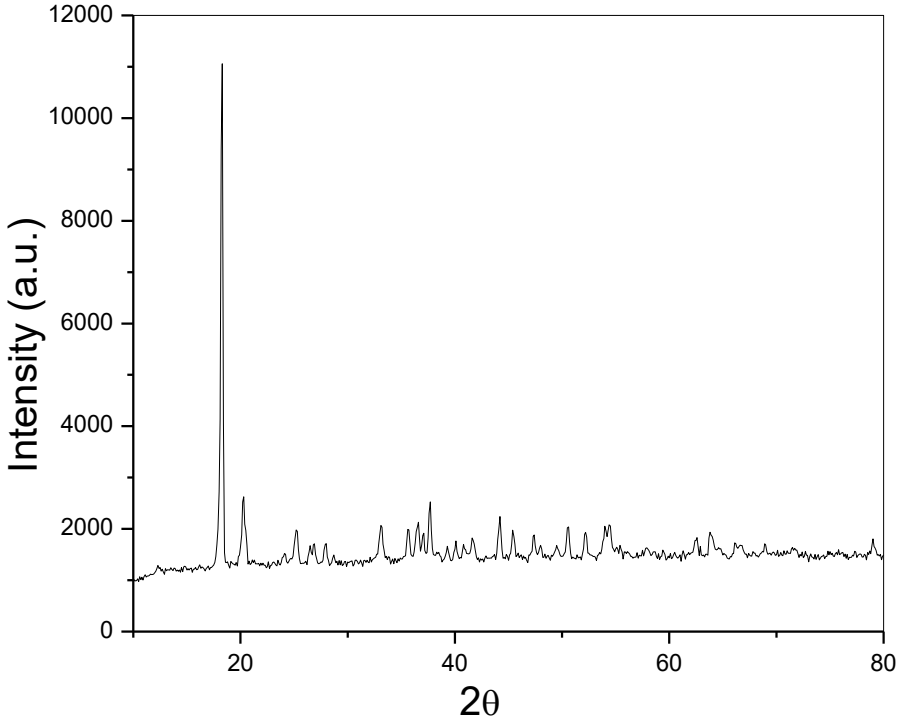

Figure 1: XRD pattern of Bauxite ore

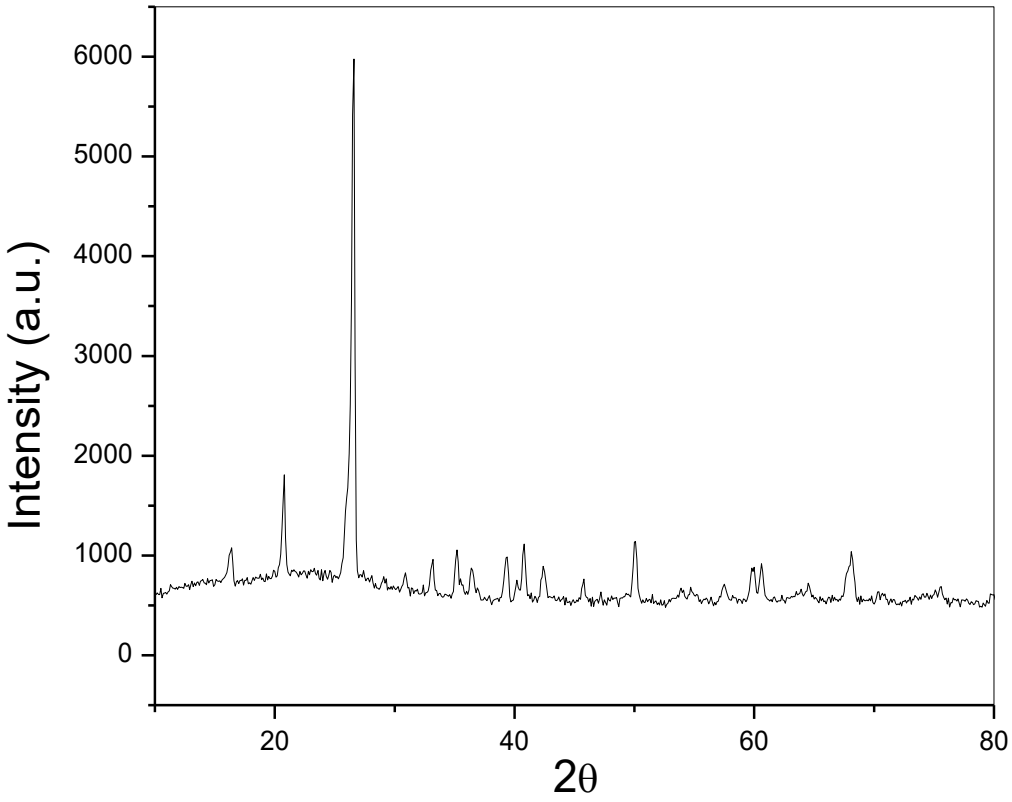

Figure 2: XRD pattern of Fly ash

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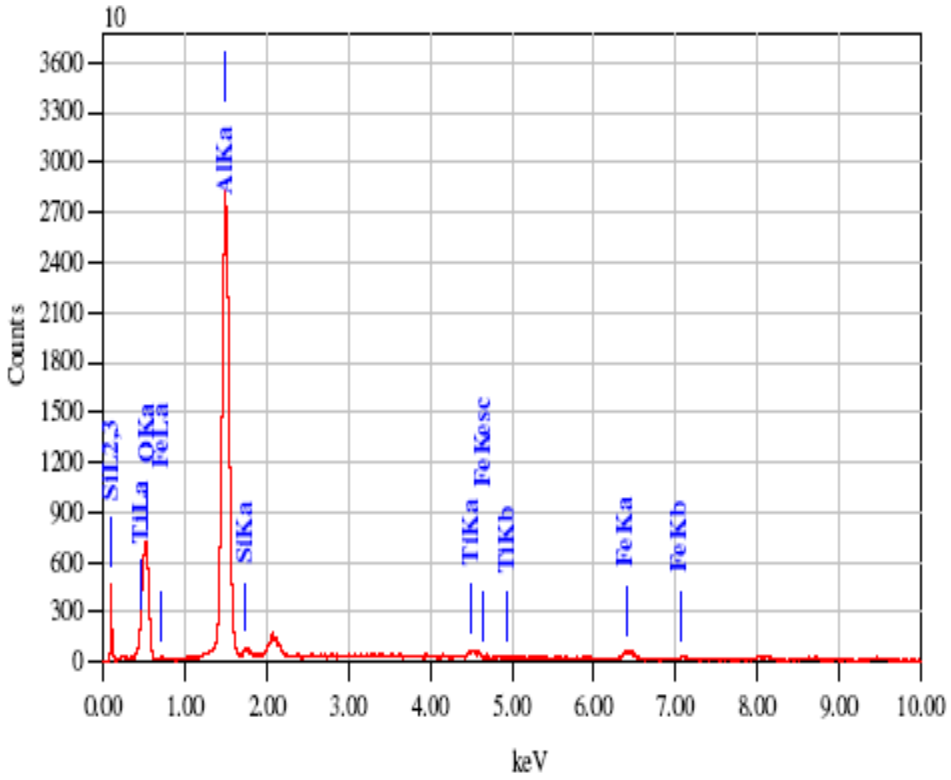

Fig.3: EDS spectrum of bauxite ore

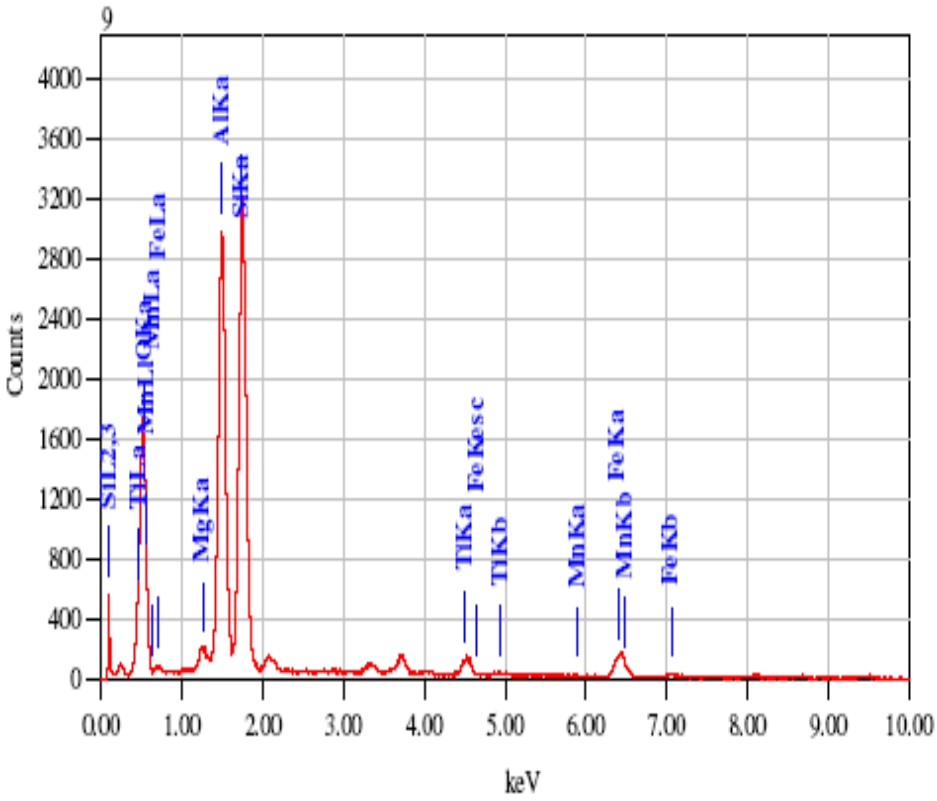

Fig.4: EDS spectrum of fly ash

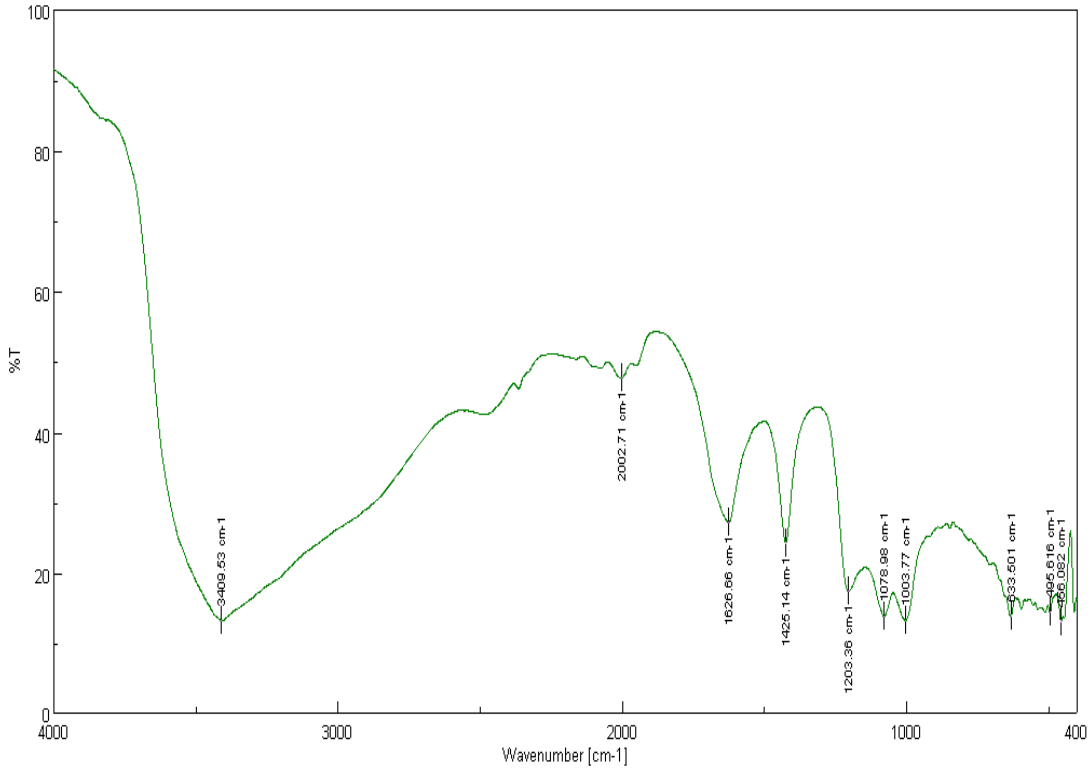

Fig: 6 FTIR spectra of bauxite ore interacted with *Thiobacillus ferrooxidans* mutant UV-26.

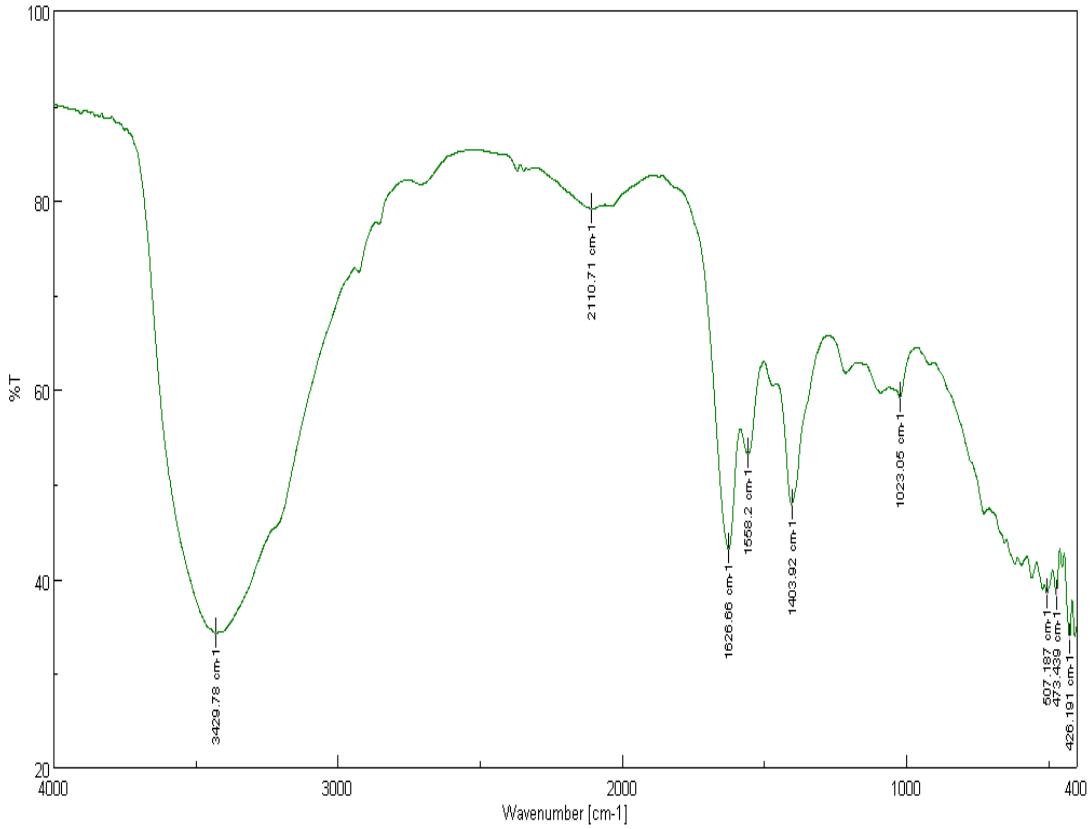

Fig: 7 FTIR spectra of untreated bauxite ore.

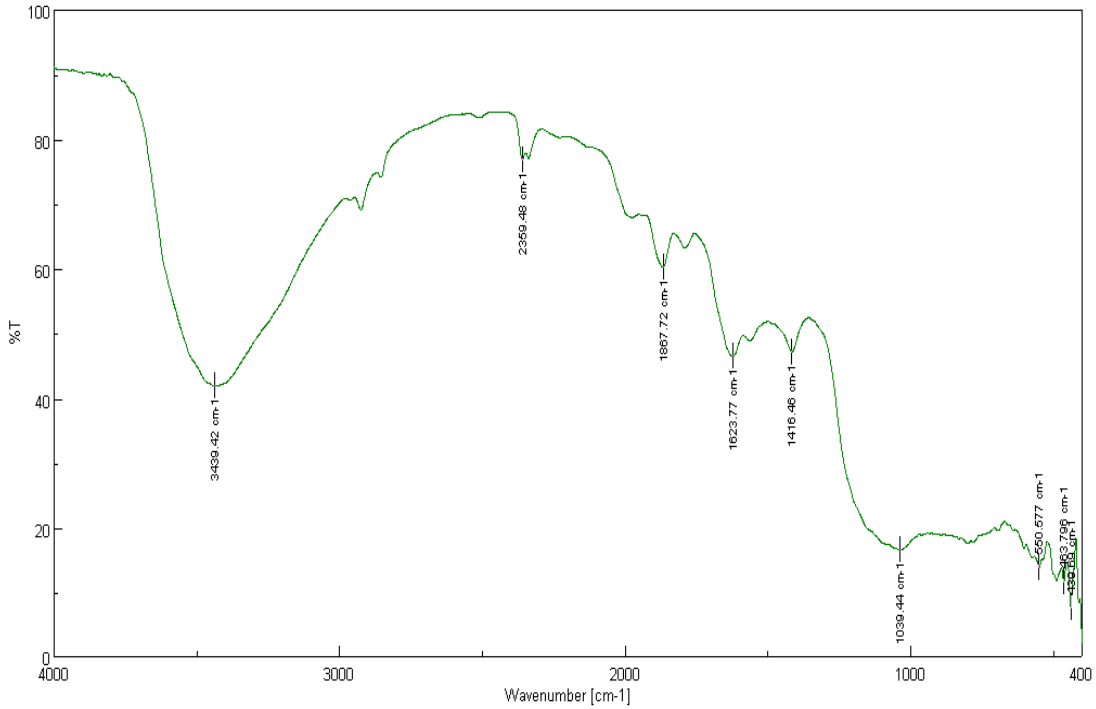

Fig: 8 FTIR spectra of fly ash interacted with *Thiobacillus ferrooxidans* mutant UV-26.

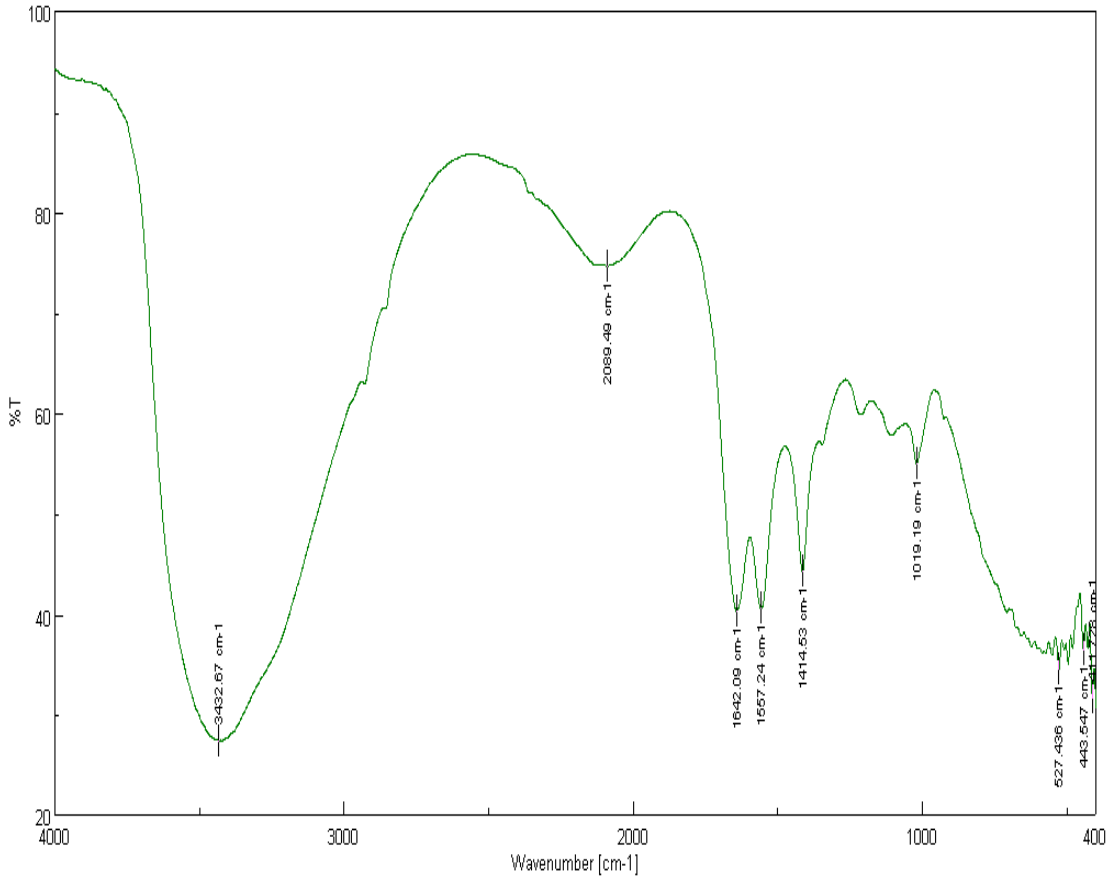

Fig: 9 FTIR spectra of untreated Fly ash.

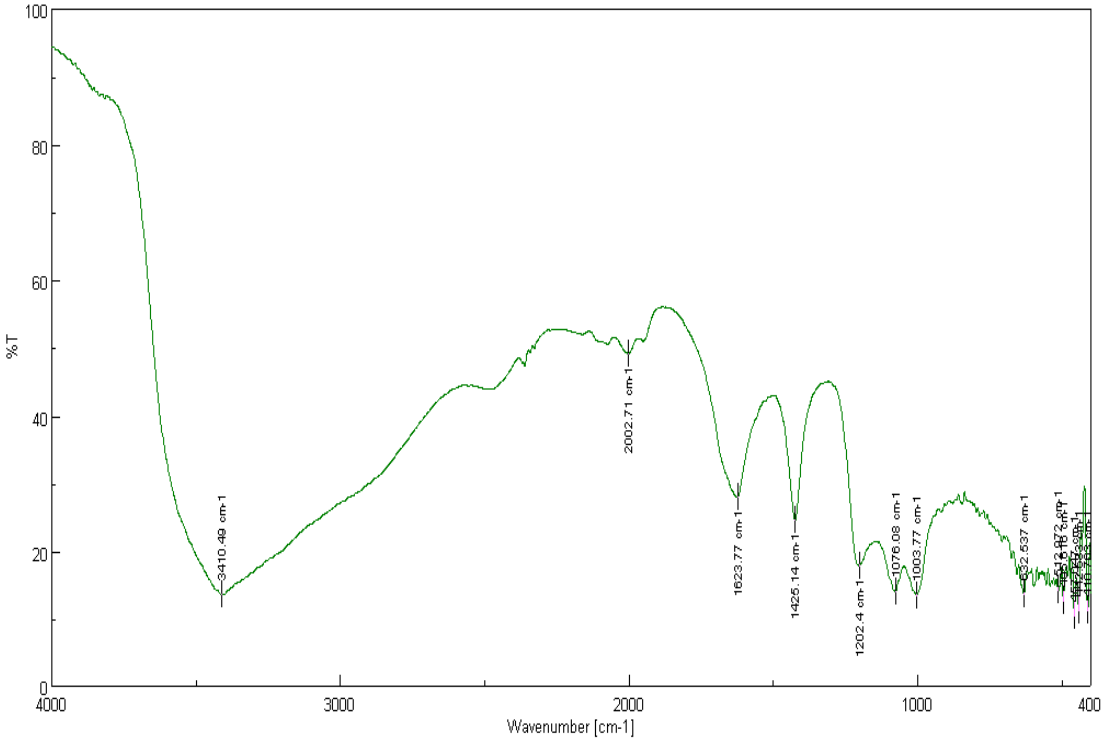

Fig 10: FTIR spectra of bauxite ore interacted with ***Pseudomonas fluorescens*** mutant UV-24

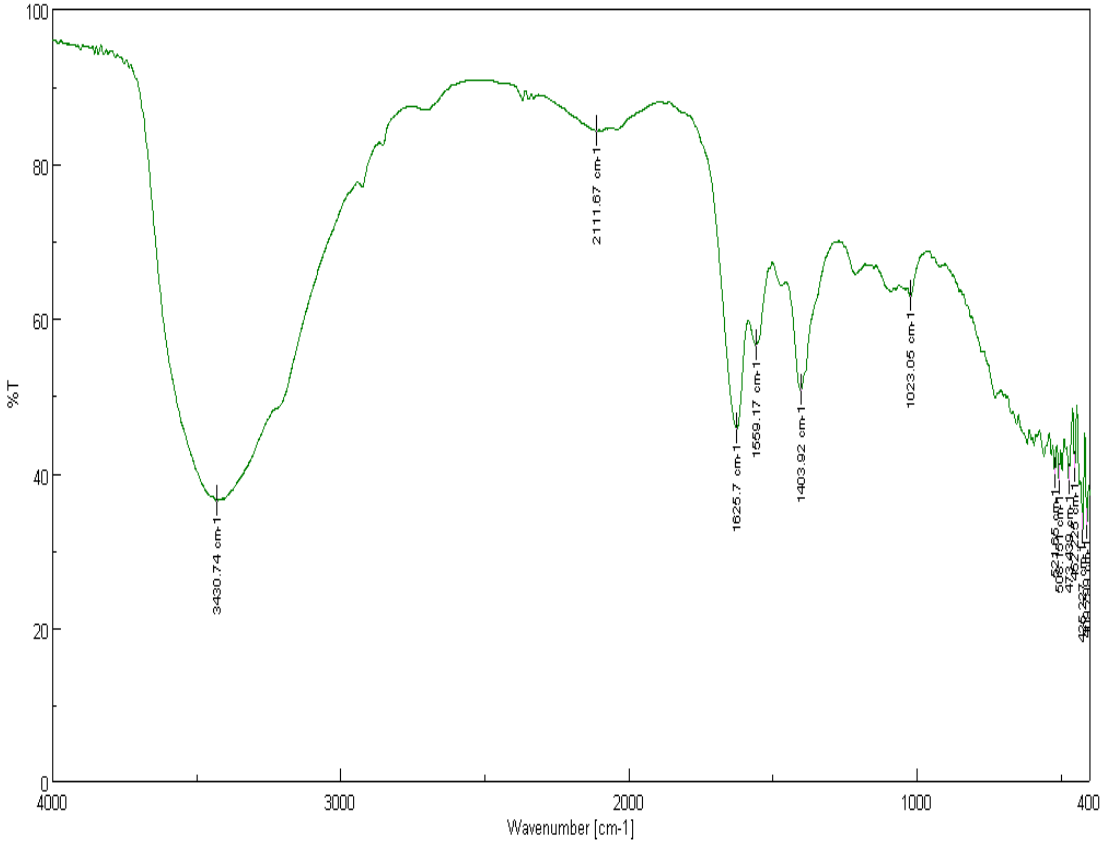

Fig 11: FTIR spectra of untreated bauxite ore

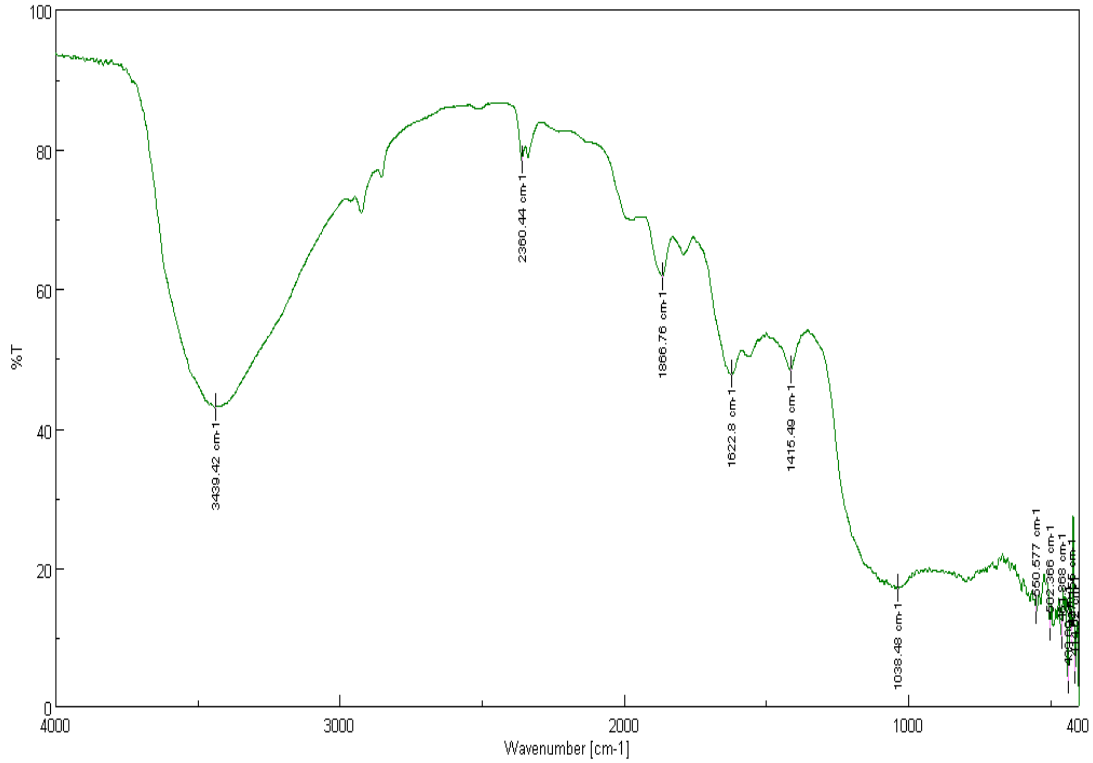

Fig 12: FTIR spectra of flyash interacted with ***Pseudomonas fluorescens*** mutant UV-24

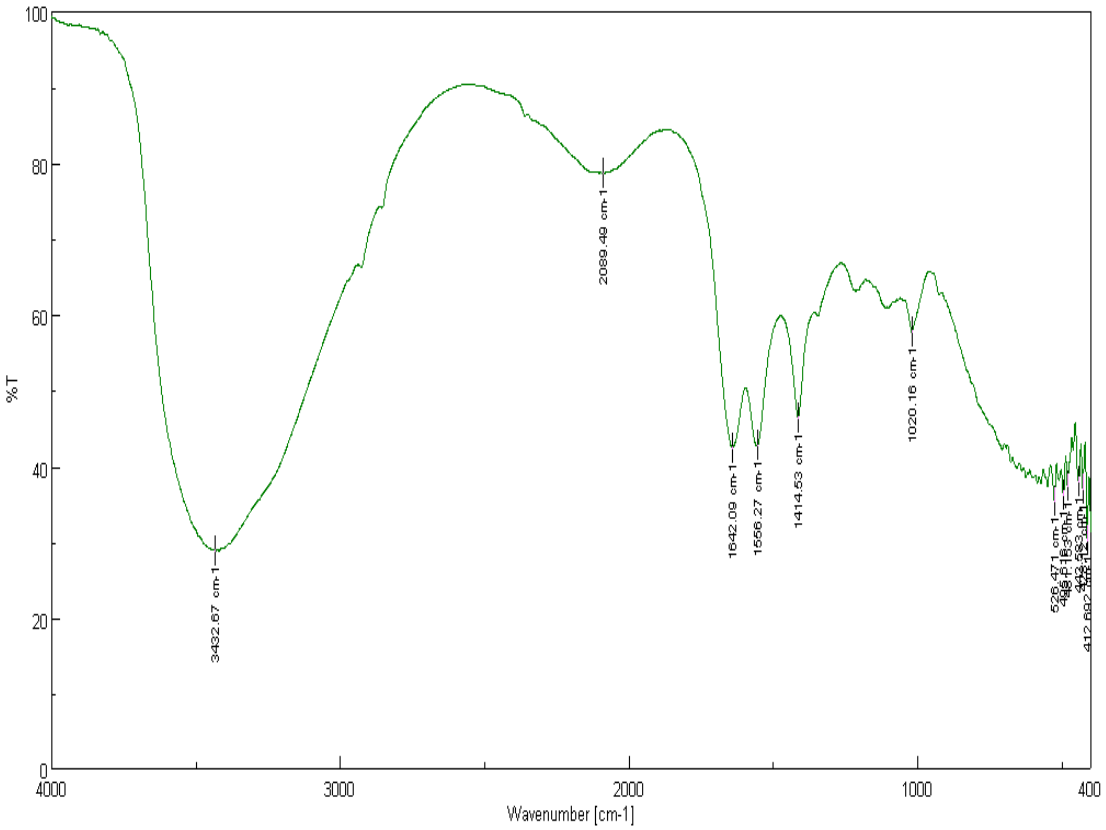

Fig 13: FTIR spectra of untreated flyash.

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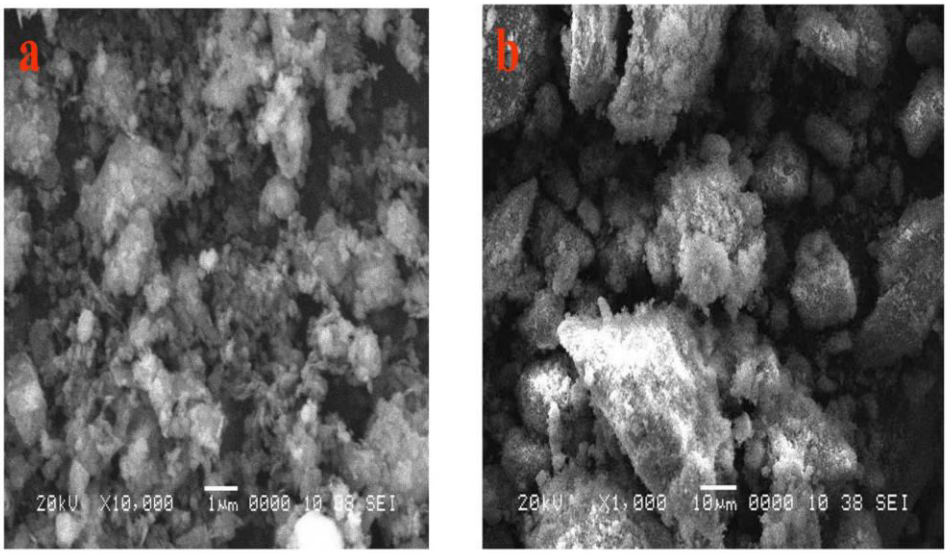
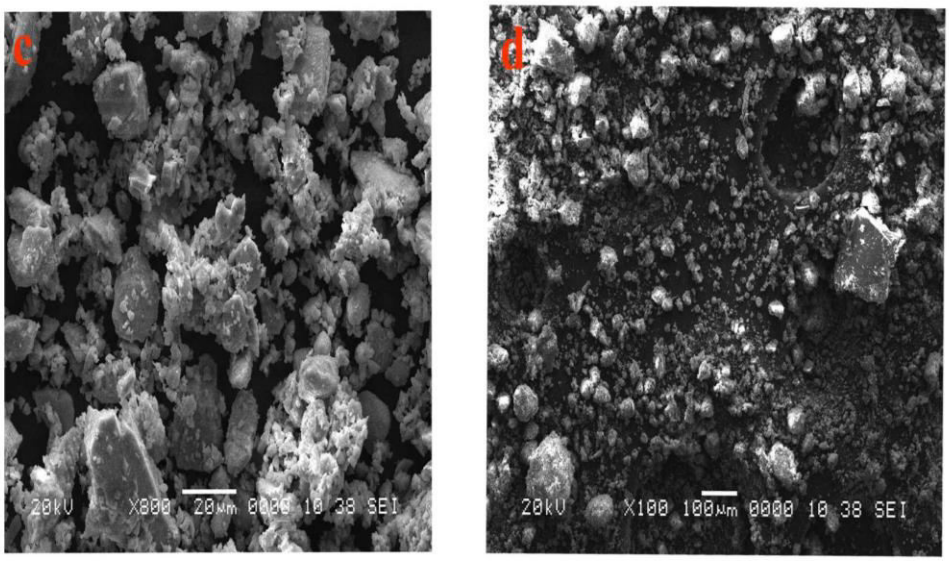
Plate I



SEM images of bauxite ore :- a) 1µm. b) 10µm.



SEM images of bauxite ore :- c) 20µm. d) 100µm.

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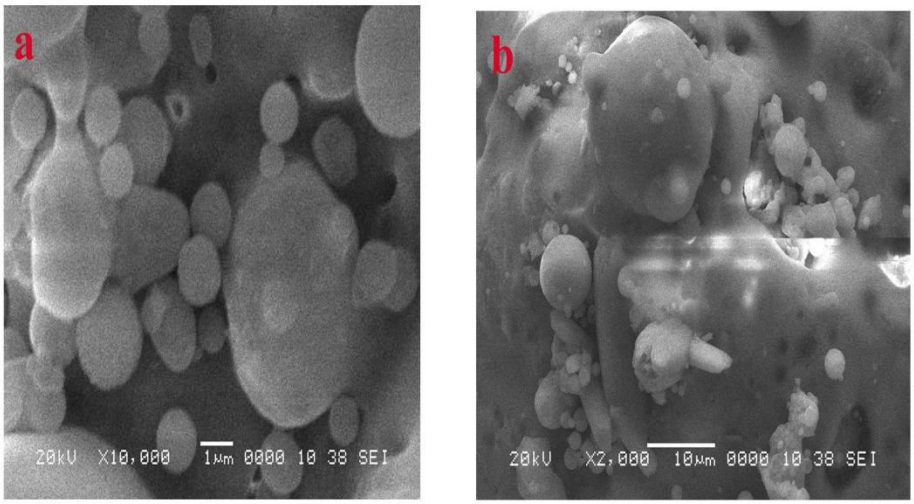
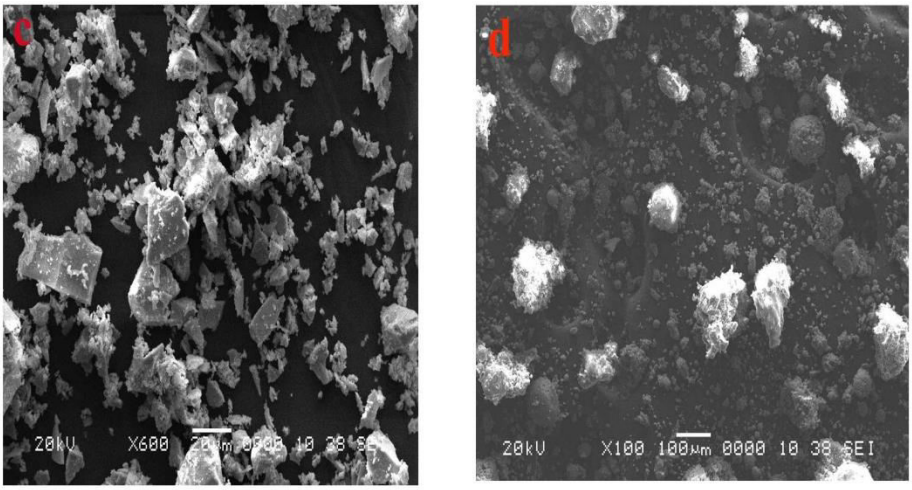
Plate II



SEM images of Fly ash: a) 1µm. b) 10 µm



SEM images of Fly ash: c) 20µm. d) 100µm.

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Plate III

a) *Thiobacillus ferrooxidans* autotrophic growth on 9K solid medium

b) *Thiobacillus ferrooxidans* chemoorganotrophic growth on solid glucose medium

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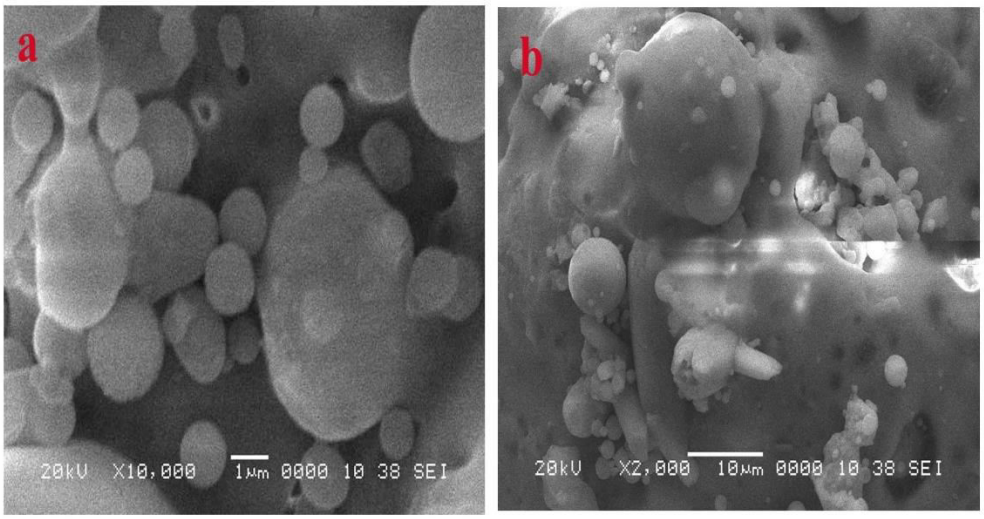
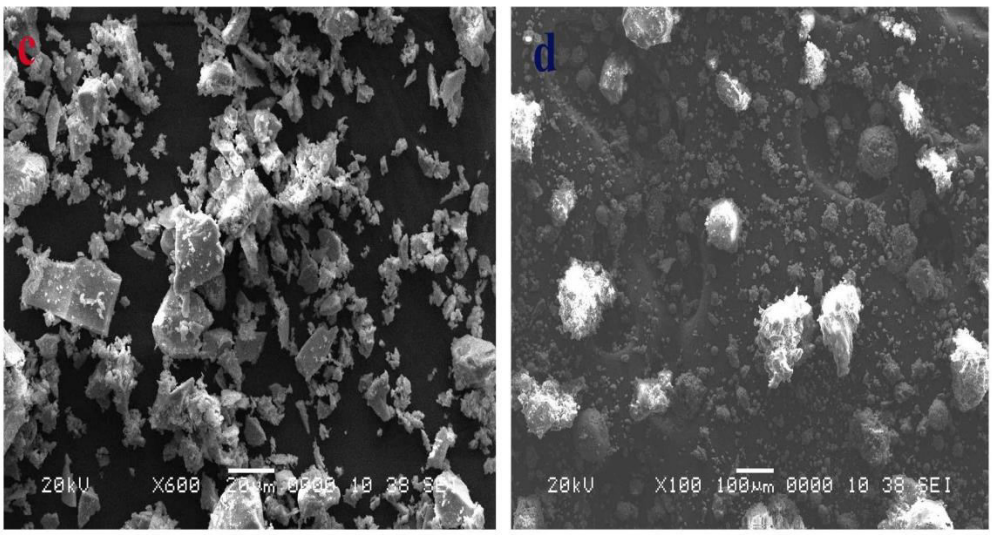
Plate III



SEM images of Fly ash: a) 1µm. b) 10



SEM images of Fly ash: c) 20µm. d) 100µm.

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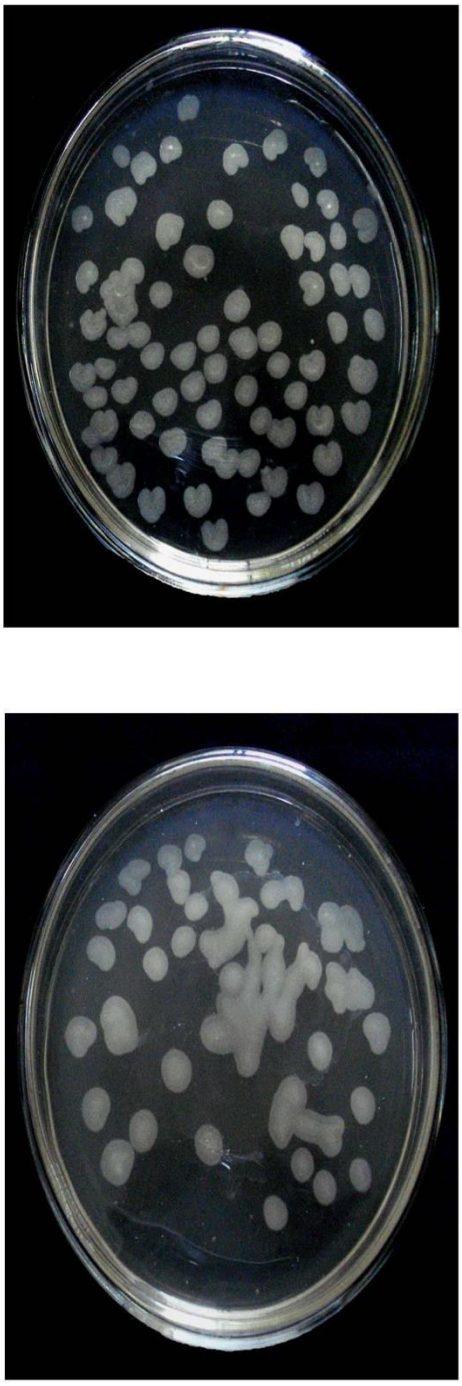
Plate IV



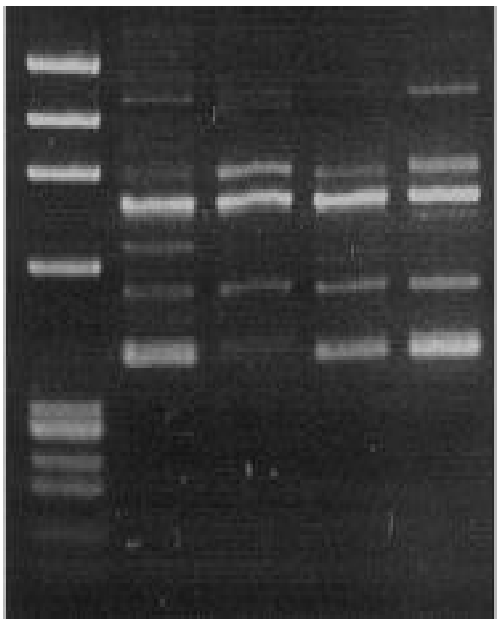
Pseudomonas fluorescens colonies growth on Kings medium

Plate V

Primer No. 861

Primer - ACC ACC ACC ACC ACC ACC

M 1 2 3 4

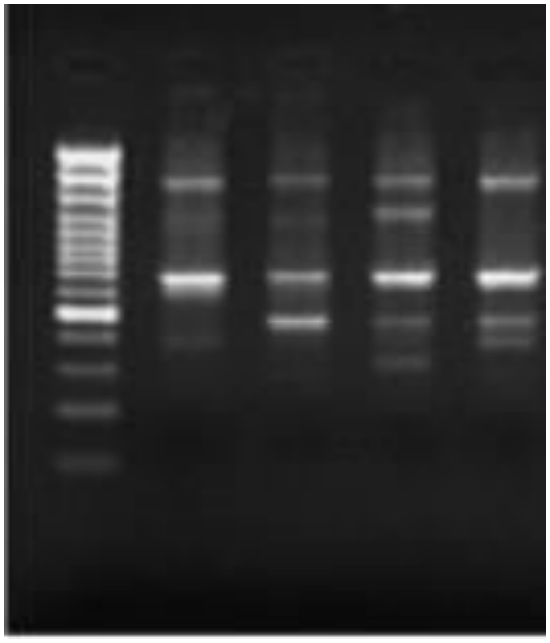


Lane M (Marker) – 100 bp DNA Marker (100 bp to 1000 bp)

Primer 862

Primer - AGC AGC AGC AGC AGC AGC

M 1 2 3 4



Lane M (Marker) – 100 bp DNA Marker (100 bp to 2000 bp)

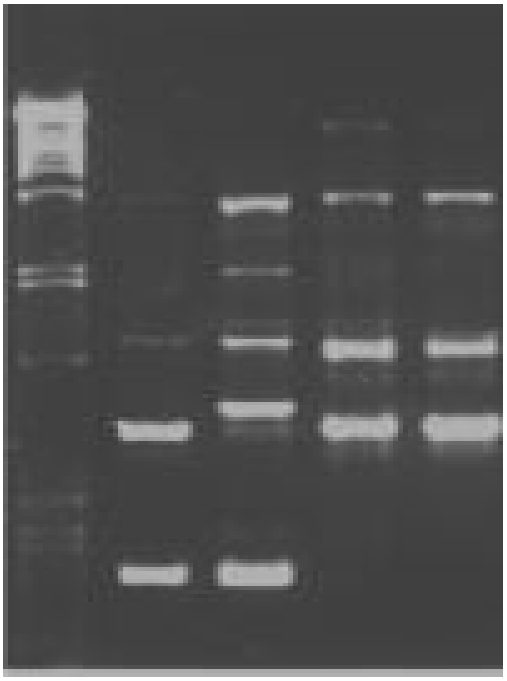
 ISBN: 978-93-93810-46-5

Plate VI

Primer No. 867

Primer - GGC GGC GGC GGC GGC GGC

M 1 2 3 4

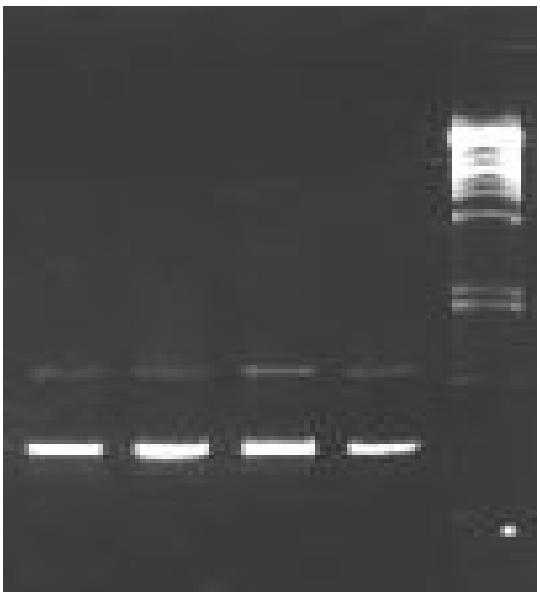


Lane M (Marker) –High Range DNA Marker (200 bp 2300 bp)

Primer No. 868

Primer - GAA GAA GAA GAA GAA GAA

1 2 3 4 M



Lane M (Marker) –High Range DNA Marker (200 bp to 2300 bp)

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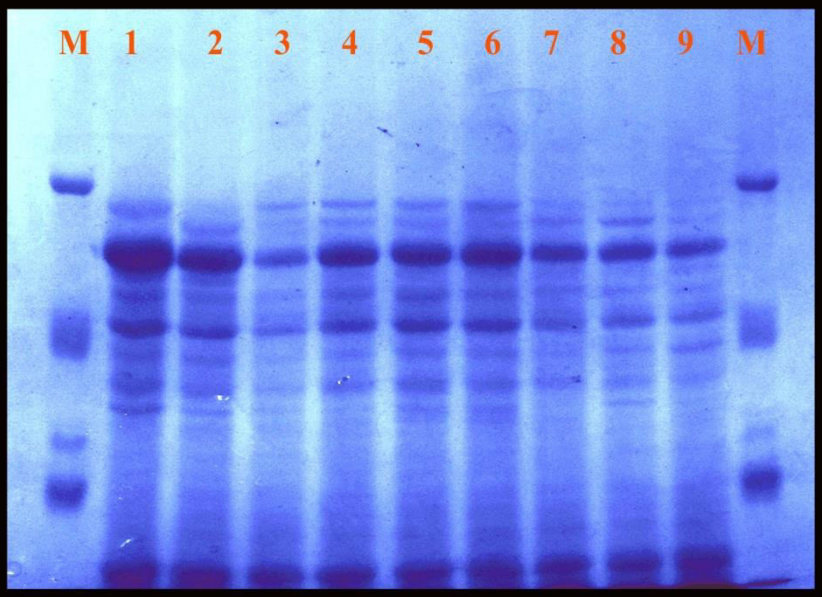
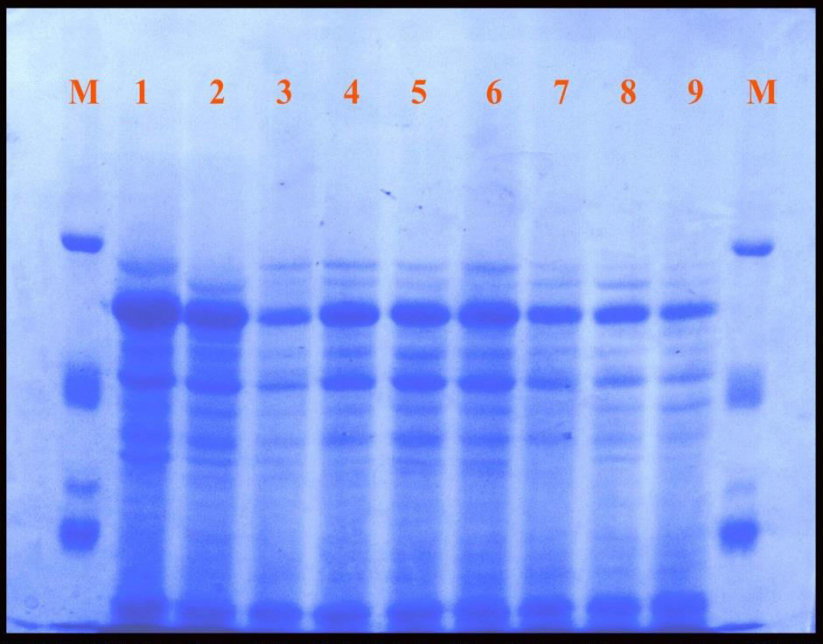
Plate VII

***a) Thiobacillus ferrooxidans* mutant UV 26**



***b) Pseudomonas fluoresecens* UV mutant 24**



SDS-PAGE Protein profile of a) Thiobacillus ferrooxidans mutant UV 26 b) *Pseudomonas fluoresecens* UV mutant 24:Lane 1 (Control) Lane 2 (1mg/ml Fe) Lane 3 (2mg/ml Fe) Lane 4(1mg/ml Al) Lane 5 (2mg/ml Al) Lane 6 (1mg/ml Si) Lane 7 (2mg/ml Si) Lane 8 (1mg/ml Ti) Lane 9 (2mg/ml Ti).

Table: 3 Physiochemical characteristics of bauxite ore and fly ash.

Parameters	Bauxite ore	Fly ash
Texture	Silty clay loam	Silt loam
Water holding capacity (%)	21.45	61.50
pH	7.5	8.38
Total carbon (%)	0.63	0.12
Porosity (%)	42.00	26.00
Total nitrogen (%)	0.062	0.0031
Phosphorus (%)	0.039	0.035
Potassium (%)	0.023	0.02
Sodium (%)	0.120	0.123
Colour	Dark red	Whitish black

Table 4: XRD analyses of bauxite ore

2Theta	Interplaner spacing	Intensity of peaks	Area under the curve	Fall width at half maximum
12.416	7.12312	45.200	849.20	1.8474
18.223	4.86430	2436.6	20132.40	0.9396
20.367	4.35680	200.6	2100.40	1.0606
25.066	3.54975	81.50	885.60	1.1666
33.068	2.70675	194.50	2673.30	1.3778
37.173	2.41676	261.90	6849.10	2.4769
41.375	2.18046	93.50	1489.80	1.6217
44.248	2.04535	140.30	2857.10	2.0409
47.557	1.91046	67.90	727.00	1.1123
50.353	1.81075	72.10	898.70	1.2375
54.341	1.68689	228.70	3405.70	1.5073
58.061	1.58736	48.50	675.20	1.4078
64.001	1.45359	121.00	2799.40	2.5306
66.542	1.40412	63.80	746.60	1.1478
71.613	1.31663	46.10	659.20	1.4250
79.048	1.21039	56.60	843.40	1.4930

Table: 5 XRD analyses of fly ash

2Theta	Interplaner spacing	Intensity of peaks	Area under the curve	Fall width at half maximum
16.236	5.45489	82.60	1060.8	1.3214
20.725	4.28238	199.70	2740.7	1.3807
26.454	3.36659	2020.20	20363.7	1.0954
33.082	2.70566	46.30	452.5	1.0455
35.525	2.52499	109.10	2478.3	2.1296
40.618	2.21935	57.60	1345.7	2.2465
42.596	2.12077	32.60	282.1	0.9326
45.751	1.98159	36.50	427.1	1.2037
50.011	1.82231	162.66	2392.8	1.4666
54.271	1.68889	63.20	1305.3	2.0010
60.230	1.53527	140.90	2254.8	1.5826
64.230	1.44895	59.20	1071.1	1.7708
67.987	1.37774	166.20	2309.1	1.4122
75.190	1.26263	45.30	950.6	2.1254
80.924	1.18701	64.80	1318.7	1.9918

Table: 6 Mineralogical analyses of bauxite ore.

Mineral identified	Mineral formula
Diaspora	$Al_2O_3\,H_2O$
Kaolinite	$Al_2\,(Si_2\,O_5)\,(OH)_4$
Gibbsite	$Al_2O_3 3H_2O$
Illite 2M#2	$KAl_2\,(Si_3\,Al)\,O_{10}\,(OH)_2$
Hematite	Fe_2O_3
Goethite	FeOOH
Boehmite	(AlOOH)
Trydymite-2OHsyn	SiO_2
Biotite -2M#1	$KMg_3\,(Si_3Al)_{10}\,(OH)_2$
Albite-disordered	$Na\,(Si_3\,Al)\,O_8$
Calcite	$(CaCO_3)$
Wallastonite	$CaSiO_3$
Anatase	TiO_2

Table: 7 Mineralogical analysis of fly ash.

Mineral identified	Mineral formula
Quartz	$(Si\,O_2)$
Coesite	(SiO_2)
Sodium aluminum silicate	$(Na\,(Al\,Si_2O_6)\,)$
Tricalcium mangnese orthosilicate	$(Ca_3\,Mn\,(SiO_4)_2)$
Magnesium silicate	$(Mg_2\,SiO_4)$
Calcium aluminum silicate	$((\,Ca_3\,Al_2\,(SiO_4)_3)$
Ferric oxide	(FeO_3)
Mullite	$(Al_4\,Si_2\,O_{10})$
Magnetite	(Fe_3O_4)
Hematite	Fe2O3

Table: 8 EDX Analysis of Bauxite Ore

Element	Kev	Mass %	Atom %
Al	1.486	46.27	35.62
Si	1.739	2.40	1.78
Ti	4.508	1.46	0.63
Fe	6.398	3.00	1.12
O	0.525	46.87	60.86
Total		100.00	100.00

Table: 9 EDX analysis of fly ash.

Element	Kev	Mass %	Atom %
Al	1.486	20.20	15.79
Si	1.739	25.04	18.80
Ti	4.508	1.99	0.87
Fe	6.398	4.24	1.60
O	0.525	46.88	61.81
Mg	1.253	1.01	0.87
Mn	5.894	0.64	0.25
Total		100.00	100.00

Table 10: Chemical estimation of metal concentration in bauxite ore

Metals	Metal concentration (mg/g)
Aluminum	364
Iron	164
Titanium	-
Silicon	-

Table 11: Chemical estimation of metal concentration in fly ash

Initial wt of fly ash (g)	Metal concentration in fly ash (mg)		
-	Al	Fe	Mn
2	120±1.22	48.32±1.60	8.00±2.00
4	246 ± 1.84	94.33±1.20	14.86±1.22
6	364 ± 1.54	144.42±2.0	24.44±1.44
8	480 ± 2.56	196.70±1.34	32.04±0.60
10	604 ± 1.40	240.40±1.60	42.66±1.60
12	724 ± 2.84	286.46±2.0	54.00±1.33

Table: 12 Quantitative analysis of fungal species at different layers of bauxite mine level

Fungal species	Fungal population in %					Relative frequency of fungal species
	Level-1 (2M)	Level-2 (4M)	Level-3 (8M)	Level-4 (16M)	Level-5 (20M)	
Aspergillus flavus	26.66	28.31	27.27	25.58	25.51	26.86
Aspergillus fumigatus	20.31	15.92	15.58	18.60	21.05	17.82
Aspergillus kanagwensis	2.33	0.88	-	-	-	1.22
Aspergillus niger	12.69	15.04	14.28	13.95	-	14.16
Aspergillus terreus	-	-	5.19	0.64	-	1.22
Fusarium oxysphorum	13.33	13.71	16.88	16.27	10.52	14.28
Cuvrularia lunata	3.17	3.53	-	-	-	2.19
Neocosmophora vasinpicta	0.62	-	-	-	5.26	0.73
Penicillium sp.	11.42	1194	16.88	20.93	10.52	13.55
Rhizopus stolonifer	8.25	3.53	3.89	4.65	10.52	5.86
Trichoderma sp.	-	2.65	-	-	-	0.73
Aspergillus nidulans	1.58	0.88	-	-	-	0.85

Table: 13 Number of fungal occurrence at various depths of bauxite mine.

Level of the mine	Depth of mine (m)	Number of fungal Colonies x 10^3	Fungal occurrence (%)
Sample - 1	2	315	38.46
Sample -2	4	226	27.59
Sample -3	8	154	18.80
Sample -4	16	86	10.50
Sample -5	20	38	4.63

Table: 14 Microbiological characteristics of fly ash and bauxite ore

Microbial groups c.f.u./g	Bauxite ore	Fly ash
Bacteria	36 x 10^2	21x10^2
Fungi	86 x 10^2	68x10^2
Actinomycetes	42 x 10^2	8x10^2

Table: 15 Physiological characteristics of *Thiobacillus ferrooxidans* isolated from bauxite ore.

Test	Response
Gram reaction	-ve
Morphology	Rod
Size	0.4-0.6 μm
Colony characters	Irregular, dark brown to yellow, raised, flat scattered.
Antibiotics resistance	Ampiciline, tetracycline, kennamycine, chlorophenicol.
Source of isolation	Bauxite ore
Autotrophic growth with Ferrous iron	+
Autotrophic growth with Thiosulphate	+
Chemoorganotrophic growth with glucose	+
Spore	-
motility	-
Growth at pH 1.8	+
Growth at pH 2.0	+
Growth at pH 2.5	+
Growth at pH 3.5	+
Growth at pH 4.0	+
Growth at pH 4.5	+
Growth at pH 7.5	+
Catalase	+
Oxidase	-
Thiosulphate	+
Glucose	+
Hydrolysis of starch	+
Citrate	-
Indole	-
Methyl red	-
Voges-Proskuer	-
Urease	_

Table: 16 Physiological characteristics of ***Pseudomonas fluorescens*** isolated from bauxite ore

Test	Response
Source of isolation	Bauxite ore
Gram reaction	-ve
Cell shape	Rods
Cell size	1 – 4 μm
Spore	-
Colony character	Regular, fluorescent, opaque, rounded, and smooth.
Anaerobic growth	-
Indole formation	-
Catalase	+
Oxidase	+
Nitrate reduction	-
Glucose fermentation	-
Urease	-
Gelatin hydrolysis	-

Citrate utilization	+
Maltose utilization	-
Growth at 5% NaCl	+

Table: 17 Effect of pH on the growth of Thiobacillus and Pseudomonas isolated from bauxite ore.

pH	**Thiobacillus ferrooxidans**	**pH**	**Pseudomonas fluorescens**
1.0	+ +	4.0	-
1.5	+ + +	5.0	+
2.0	+ + +	6.0	+ +
2.5	+ + +	7.0	+ + +
3.0	+ +	8.0	+ + +
4.0	+ +	9.0	+ +
5.0	+ +	10.0	+
6.0	+ +	11.0	-
6.5	+	12.0	-
7.0	-		

Table: 18 Effect of NaCl concentrations on the growth of Thiobacillus and Pseudomonas isolates isolated from bauxite ore.

.pH	***Thiobacillus ferrooxidans***	**Pseudomonas fluorescens**
0.5	+ + +	+ + +
1.0	+ + +	+ + +
1.5	+ + +	+ + +
2.0	+ + +	+ + +
2.5	+ + +	+ + +
3.0	+ + +	+ + +
4.5	+ +	+ +
5.0	+	+

Table: 19 Growth of Thiobacillus and Pseudomonas isolates at different temperature.

Temperature in (^{o}C)	***Thiobacillus ferrooxidans***	**Pseudomonas fluorescens**
10	+	+
15	+	+
20	+	+
25	+ +	+ +
30	+ + +	+ + +
35	+ + +	+ + +
40	+ + +	+ +
45	+ +	+ +
50	+ +	+

Table: 20 Oxidation rate of ferrous ions in presence of bauxite ore and fly ash by *Thiobacillus ferrooxidans* mutant.

Incubation time	**Per cent ferrous ion oxidation**		
	Control	**Bauxite ore**	**Fly ash**
0.00	0.00	0.0	0.0
12.0	8.0	6.5	5.8

24.0	24.0	22.8	20.5
36.0	40.0	36.4	32.8
48.0	46.0	40.5	39.4
60.0	62.0	48.2	45.8
72.0	68.0	62.4	61.2
84.0	76.0	68.5	66.0
96.0	82.0	72.8	70.8
108	86.0	78.5	75.2
120	92.0	82.4	79.5

Table: 21 Growth of ***Pseudomonas fluorescens*** mutant in presence of bauxite ore and fly ash.

Incubation time (hours)	**Optical density at 660nm.**		
	Control	**Bauxite ore**	**Fly ash**
1.00	0.2	0.2	0.1
2.00	0.4	0.3	0.3
4.00	0.8	0.6	0.7
6.00	1.2	1.0	0.9
8.00	1.4	1.2	1.0
10.0	1.5	1.4	1.2
12.0	1.7	1.6	1.4
18.0	1.9	1.8	1.6
24.0	1.4	2.0	1.8
36.0	1.3	1.6	1.9
40.0	1.2	1.4	1.4

Table: 22 Effect of various metal ion concentration on growth of *Thiobacillus ferrooxidans*

Concentration of metals (mg/ml)	**Growth in O.D. at 660 nm**			
	Titanium	**Silicon**	**Aluminium**	**Iron**
0	2.26	2.46	2.34	2.1
1000	2.2	2.36	2.08	2.02
2000	2.14	2.2	1.98	1.86
3000	2.08	2.08	1.84	1.72
4000	1.98	1.98	1.62	1.42
5000	1.86	1.66	1.46	1.04

Table: 23 Effect of various metal ion concentration on growth of **Pseudomonas fluorescens**

Concentration of metals (mg/ml)	**Growth in O.D. at 660 nm**			
	Titanium	**Silicon**	**Aluminium**	**Iron**
0	2.2	2.85	2.4	2.86
1000	2.02	2.42	2.3	2.4
2000	1.86	2.1	2.06	2.26
3000	1.2	1.68	1.84	1.98
4000	0.86	1.08	1.65	1.72
5000	2.45	0.64	1.02	1.02

Table: 24 Effect of adapted and non-adapted strains of Thiobacllus ferrooxidans on bioextraction of metals from bauxite ore

Microorganism Used.	Bauxite ore (g)		Metal extracted (mg)		Metal extracted %		
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	Bioextraction Efficiency of Al and Fe (%)
Adapted *Thiobacillus ferrooxidans*	2	2.924	578.67±1.72	268.27±0.23	79.48	81.78	80.20
Non-adapted *Thiobacillus ferrooxidans*	2	2.847	542.66±1.64	226.67±0.56	74.54	69.10	72.85
Control	2	2.00	4.54±1.22	5.66±1.44	0.623	1.72	0.965
C.D. (p=0.05)			46.27	53.46			

Table: 25 Effect of adapted and non-adapted strains of ***Pseudomonas fluorescens*** on bioextraction of metals from bauxite ore

Microorganism Used.	Bauxite ore (g)		Metal extracted (mg)		Metal extracted %		
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	Bioextraction Efficiency of Al and Fe (%)
Adapted **Pseudomonas fluorescens**	2	2.217	186.66±2.78	125.78±1.59	25.64	38.34	29.58
Non-adapted pseudomonas fluorescens	2	2.196	159.33±2.27	118.45±1.47	21.88	36.11	26.3
Control	2	1.986	2.66±0.86	3.22±0.98	0.84	0.98	0.55
C.D. (p=0.05)			35.12	5.42			

Table 26: Development of mutants through induced mutagenesis

Microbes	Mutagenic agents	Colony Forming Units			Mutation frequency	
		Control	10mg/ml	20mg/ml	10mg/ml	20mg/ml
Pseudomonas fluorescens	EMS	$286x10^2$	$186x10^2$	$124X10^2$	65.03	43.35
	UV	$246x10^2$	126x102	$84x10^2$	51.21	34.14
Thiobacillus ferrooxidans	EMS	$86x10^2$	$68x10^2$	$48x10^2$	79.06	55.81
	UV	$94x10^2$	$82x10^2$	$64x10^2$	76.59	68.08

Table 27: Extraction of metals from bauxite ore by UV mutants of *Thiobacillus ferrooxidans*

UV- Isolates	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
UV -isolate 1	2	2.789	509.33±2.25	178.34±1.47	69.96	54.37	65.12
UV- isolate 2	2	2.868	543.33±3.64	221.13±2.45	74.63	67.41	72.39
UV - isolate 3	2	2.523	413.00±2.59	184.98±2.70	56.73	56.39	56.62
UV - isolate 4	2	2.645	470.00±1.11	223.32±2.78	64.56	68.08	65.65

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UV - isolate 5	2	2.546	438.33±2.64	108.23±1.42	60.21	32.99	51.75
UV - isolate 6	2	2.684	508.32±2.64	168.23±1.43	69.82	51.33	64.06
UV - isolate 7	2	2.787	535.00±1.12	196.65±2.43	73.48	59.95	69.28
UV - isolate 8	2	2.968	581.66±2.64	123.45±1.08	79.89	37.63	66.77
UV - isolate 9	2	2.764	535.40±1.14	178.41±1.56	73.48	54.39	67.55
UV- isolate 10	2	2.723	532.30±1.23	167.30±2.42	73.07	51.00	66.22
UV- isolate 11	2	2.816	540.80±1.34	198.40±2.76	74.17	60.48	69.92
UV- isolate 12	2	2.867	565.00±1.43	182.45±1.23	77.60	55.62	70.78
UV- isolate 13	2	2.684	248.36±1.70	186.23±2.46	34.11	56.77	41.15
UV- isolate 14	2	2.564	448.21±2.64	184.00±1.06	61.56	56.09	59.86
UV- isolate 15	2	2.846	351.66±1.70	176.87±2.34	48.30	53.92	50.05
UV- isolate 16	2	2.843	450.00±2.95	221.68±1.68	61.81	67.58	63.60
UV- isolate 17	2	2.865	561.64±3.68	234.23±2.78	77.14	71.41	75.36
UV- isolate 18	2	2.805	238.00±1.45	147.23±1.63	32.69	44.88	36.48
UV- isolate 19	2	2.685	305.32±1.13	181.82±0.65	41.89	55.43	46.10
UV- isolate 20	2	2.801	336.68±1.29	190.37±1.45	46.24	58.03	49.91
UV- isolate 21	2	2.453	462.46±2.44	182.98±2.47	63.46	25.29	51.60
UV- isolate 22	2	2.802	541.63±2.64	187.31±2.56	74.39	57.10	69.02
UV- isolate 23	2	2.763	523.78+±2.67	72.12±1.43	71.94	21.98	56.42
UV- isolate 24	2	2.594	483.32±2.64	186.34±2.78	66.39	56.81	63.41
UV- isolate 25	2	2.597	402.37±2.68	124.45±1.67	55.27	37.94	49.88
UV- isolate 26	2	2.873	593.33±2.64	278.43±2.68	81.50	84.88	82.55
UV- isolate 27	2	2.563	410.21±1.13	221.56±2.78	56.31	67.54	59.80
UV- isolate 28	2	2.783	515.22±1.12	189.56±2.45	70.74	57.79	66.71
UV- isolate 29	2	2.441	413.16±2.59	162.00±2.47	56.73	49.39	54.45
UV- isolate 30	2	2.287	265.44±2.62	221.45±2.48	36.40	67.37	46.06
Control	2	2.872	542.32+0.59	264.43±0.74	74.49	80.61	76.39
C.D. (p=0.05)			46.42	19.48			

Table 28: Extraction of metals from bauxite ore by EMS mutants of *Thiobacillus ferrooxidans*

EMS Isolates	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
Control	2	2.845	542.63±2.60	196.32±1.47	74.49	59.85	69.97
Isolate -1	2	2.823	538.08±1.44	218.34±1.48	73.90	66.56	71.63
Isolate -2	2	2.765	505.36±2.68	178.12±1.87	69.41	54.30	64.72
Isolate -3	2	2.276	278.20±3.47	246.00±1.78	38.18	75.00	49.64
Isolate -4	2	2.734	508.00±2.47	172.34±1.43	69.78	52.54	64.42
Isolate-5	2	2.467	274.31±2.84	197.23±2.68	37.63	60.13	44.65
Isolate-6	2	2.721	452.14±1.46	182.65±2.47	62.08	55.68	60.11
Isolate-7	2	2.443	294.66±1.36	208.00±1.23	40.47	63.41	47.6
Isolate -8	2	2.213	137.68±1.58	134.78±2.45	18.91	41.09	25.8
Isolate -9	2	2.702	430.22±1.47	174.23±2.76	59.06	53.11	57.23
Isolate -10	2	2.698	432.83±2.56	187.23±1.67	59.38	57.08	58.71
Isolate -11	2	2.296	288.36±2.49	156.46±1.47	39.60	47.69	42.12
Isolate -12	2	2.764	464.33±2.72	196.43±1.74	63.78	59.88	62.57
Isolate -13	2	2.607	383.64±4.72	266.45±2.89	52.70	81.23	61.56
Isolate -14	2	2.492	285.63±4.59	221.23±2.43	39.20	67.44	47.99

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Isolate-15	2	2.403	236.00±3.24	187.34±2.78	32.41	57.11	40.08
Isolate-16	2	2.643	385.60±1.08	266.67±2.47	52.88	81.23	61.76
Isolate -17	2	2.867	530.33±1.53	187.66+±0.48	72.86	57.11	67.99
Isolate -18	2	2.645	386.64±2.82	261.66±1.34	53.10	79.77	61.39
Isolate -19	2	2.198	257.83±2.89	142.00±2.87	35.41	43.29	37.86
Isolate -20	2	2.768	480.67±2.68	162.22±2.43	66.02	49.45	60.87
Isolate -21	2	2.478	266.45±2.36	233.43±0.42	36.60	71.16	47.33
Isolate -22	2	2.953	585.33±2.28	281.44±2.47	80.40	85.80	82.02
Isolate -23	2	2.789	511.09±1.84	143.43±2.45	70.19	43.72	61.98
Isolate -24	2	2.765	467.46±1.64	167.43±1.45	64.21	51.04	60.12
Isolate -25	2	2.798	484.66±2.82	121.42±1.48	66.57	37.01	57.39
Isolate -26	2	2.889	546.87±2.62	248.32±1.34	75.11	75.70	75.3
Isolate -27	2	2.734	427.34±2.76	168.22±1.64	58.7	51.28	56.39
Isolate -28	2	2.412	225.00±2.62	164.23±1.54	30.90	50.07	36.85
Isolate-29	2	2.723	424.65±2.49	221.45±1.45	58.33	67.51	61.18
Isolate- 30	2	2.487	265.04±2.62	187.54±2.43	36.40	57.17	42.85
C.D. (p=0.05)			55.01	10.65			

Table 29: Extraction of metals from bauxite ore by UV mutants of **Pseudomonas fluorescens**

UV Isolates	Bauxite Ore in (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
Control	2	2.346	236.45±2.60	167.62±1.66	32.47	51.11	38.26
Isolat-1	2	2.286	165.33±1.27	123.31±1.98	22.71	37.6	27.33
Isolate-2	2	2.468	291.66±2.68	130.65±2.76	40.06	39.83	39.99
Isolate-3	2	2.323	211.66±1.68	183.58±2.67	29.07	55.99	37.43
Isolate-4	2	2.172	84.66±1.98	50.00±2.47	11.62	15.24	12.75
Isolate-5	2	2.174	185.00±1.08	67.20±1.43	25.41	20.42	23.86
Isolate-6	2	2.268	144.33±1.95	137.45±0.58	19.82	41.9	26.68
Isolate-7	2	2.292	181.66±2.68	106.10±1.94	24.95	32.31	27.24
Isolate-8	2	2.336	191.66±2.68	144.34±2.96	26.32	44	31.81
Isolate-9	2	2.296	208.33±2.68	191.40±2.84	28.61	58.23	37.81
Isolate10	2	2.202	96.33±1.36	86.66±1.98	13.23	26.42	17.32
Isolate11	2	2.289	162.00±1.24	110.45±1.76	22.25	33.53	25.75
Isolate12	2	2.346	225.30±1.17	164.33±2.98	30.96	50.1	36.89
Isolate13	2	2.268	170.45±1.17	186.45±1.24	23.35	56.84	33.79
Isolate14	2	2.145	68.66±0.72	52.66±0.72	9.43	16.05	11.48
Isolate15	2	2.96	47.85±1.47	83.00±0.98	6.45	25.3	12.39
Isolate16	2	2.372	265.00±1.17	222.22±2.81	36.4	67.68	46.17
Isolate17	2	2.432	255.80±2.07	228.00±2.54	35.02	69.51	45.81
Isolate18	2	2.214	132.66±2.72	123.07±1.67	18.22	37.52	24.21
Isolate19	2	2.276	145.36±1.07	163.44±1.18	19.91	49.82	29.24
Isolate20	2	2.482	292.66±2.72	178.00±1.24	40.2	54.26	44.57
Isolate21	2	2.368	264.33±3.95	182.66±1.72	36.3	55.68	42.32
Isolate22	2	2.187	125.66±1.49	126.12±1.34	17.26	38.45	23.84
Isolate23	2	2.163	115.40±1.17	123.76±1.67	15.79	37.5	22.64
Isolate24	2	2.696	376.33±2.36	340.23±2.47	51.69	79.94	60.46

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C.D. (p=0.05)			40.44	32.42			

Table 30: Extraction of metals from bauxite ore by EMS mutants of **Pseudomonas fluorescens**

EMS Isolates	Bauxite Ore in (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
Normal strain	2	2.346	236.451±.60	167.62±1.66	32.47	51.11	38.26
Isolate-1	2	2.122	63.63±2.98	76.65±1.98	8.74	22.36	13.28
Isolate-2	2	2.322	216.66±1.44	219.33±1.72	29.76	66.86	41.28
Isolate-3	2	2.196	126.00±1.24	106.78±2.43	17.3	32.55	22.04
Isolate-4	2	2.387	244.32±2.95	182.89±2.94	33.56	55.58	40.45
Isolate-5	2	2.187	91.33±1.95	89.33± 1.79	12.54	27.23	17.1
Isolate-6	2	2.298	195.40±1.70	165.66±1.45	26.84	50.5	34.19
Isolate-7	2	2.308	209.0±1.84	129.34±1.84	28.7	39.43	32.02
Isolate-8	2	2.108	52.00±1.94	45.00±1.78	7.22	13.71	9.18
Isolate-9	2	2.396	283.66±2.95	246.76±0.98	38.96	75.23	50.22
Isolate-10	2	2.298	182.66±1.51	169.45±1.45	25.09	51.66	33.34
Isolate-11	2	2.423	283.66±1.75	243.32±1.44	25.22	74.18	40.43
Isolate-12	2	2.34	220.00±1.17	210.5±1.44	30.21	64.17	40.76
Isolate-13	2	2.321	212.68±1.18	192.00±1.47	29.21	58.53	38.32
Isolate-14	2	2.478	292.23±2.95	182.66±1.72	40.15	55.68	44.98
Isolate-15	2	2.31	186.22±1.95	68.00±1.34	25.59	20.73	24.08
Isolate-16	2	2.206	128.40±1.95	85.76±1.70	17.62	26.14	20.27
Isolate-17	2	2.314	183.63±1.98	105.67±2.63	25.18	32.21	27.36
Isolate-18	2	2.168	71.66±1.89	112.34±2.59	9.84	34.24	17.42
Isolate-19	2	2.234	148.33±1.98	89.00±1.84	20.37	27.13	22.47
Isolate-20	2	2.584	323.66±2.75	283.33±1.44	44.45	86.38	57.48
Isolate-21	2	2.108	155.00±1.70	71.00±1.86	21.29	21.64	21.4
Isolate-22	2	2.324	217.17±1.51	179.33±2.98	29.85	54.67	37.56
Isolate-23	2	2.262	142.00±1.84	92.3±11.34	19.5	28.14	22.19
Isolate -24	2	2.164	84.27±1.95	76.45±1.70	11.66	23.3	15.22
C.D. (p=0.05)			40.46	34.02			

Table 31: Effect of media composition on extraction of metals from bauxite ore by *Thiobacillus ferrooxidans*

Media used	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		
	Initial pH	Final pH	Aluminum	Iron	Aluminum	Iron	Bioextraction Efficiency (%)
Basal Salt medium	6.5	4.2	502.00±1.87	221.33±1.44	68.95	67.47	68.49
Marsh and Norris	6.5	3.4	280.44±1.17	163.34±1.72	38.52	49.79	42.02

9K with glucose	2.5	1.4	509.22±1.06	210.66±1.98	69.94	64.22	68.17
T and K Medium.	1.8	1.2	547.66±0.59	246.60±1.36	75.22	75.18	75.21
9 K without Glucose.	2.5	1.2	565.36±1.17	264.33±1.44	77.65	80.58	78.56
S.E.			51.49	17.27			
C.D. (p=0.05)			132.33	44.28			

Table 32: Effect of pH on extraction of metals from bauxite ore by *Thiobacillus ferrooxidans*

pH	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
1.0	2	2.789	482.20±3.43	262.45±1.78	66.2	80.01	70.49
2.0	2	2.896	590.66±2.78	278.23±1.26	81.13	84.82	82.28
3.0	2	2.846	564.87±2.45	264.53±1.68	77.59	80.64	78.54
4.0	2	2.856	562.10±2.56	246.67±1.45	68.95	75.2	70.89
5.0	2	2.734	465.00±1.78	216.23±1.83	63.87	65.92	64.51
6.0	2	2.72	405.33±2.67	196.43±1.64	55.67	59.88	56.98
7.0	2	2.679	384.00±2.56	178.67±2.45	52.74	54.47	53.28
8.0	2	2.523	298.08±2.43	162.00±2.34	40.12	49.39	43.56
C.D. (p=0.05)			97.57	39.97			

Table 33: Effect of pH on extraction of metals from bauxite ore by **Pseudomonas fluorescens**

pH	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
4.0	2	2.246	172.34±1.94	136.43±1.36	23.67	41.59	29.23
5.0	2	2.296	184.56±1.92	142.80±2.46	25.35	43.53	31
6.0	2	2.367	202.67±1.16	187.40±1.56	27.74	57.13	36.93
7.0	2	2.487	265.33±1.82	236.62±1.87	36.44	72.14	47.53
8.0	2	2.548	246.45±1.91	248.23±2.46	33.85	75.67	46.84
9.0	2	2.486	226.33±0.72	278.43±2.34	31.08	54.39	38.32
10.0	2	2.387	184.68±0.94	189.78±1.36	25.36	57.85	35.46
11.0	2	2.289	124.66±0.36	152.23±1.78	17.07	46.41	26.22
12.0	2	2.176	102.33±0.72	106.43±1.54	14.05	32.44	19.76
C.D. (p=0.05)			45.60	49.57			

Table 34: Effect of temperature on extraction of metals from bauxite ore by *Thiobacillus ferrooxidans*

Temperature	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe
	Initial	Final	Aluminum	Iron	Aluminum	Iron	

	wt	wt					(%)
4 ^{0}C	2	2.87	23.00±1.72	16.42±0.47	3.15	5	3.73
10^{0}C	2	2.96	39.66±1.49	26.23±0.42	5.44	7.99	6.23
20^{0}C	2	2.123	94.33±1.76	76.00±0.92	12.95	23.17	16.12
24^{0}C	2	2.167	124.66±1.36	96.08±0.72	17.12	29.29	20.9
28^{0}C	2	2.423	244.43±2.47	192.47±1.82	33.57	58.67	41.37
32^{0}C	2	2.621	384.00±2.23	261.22±2.34	52.74	79.64	61.10
36^{0}C	2	2.878	596.34±3.23	278.44±1.47	81.91	84.89	82.83
40^{0}C	2	2.864	587.36±2.36	242.46±1.92	80.68	73.92	78.58
44^{0}C	2	2.846	574.67±2.76	221.66±2.90	78.93	67.57	75.41
48^{0}C	2	2.71	524.33±2.47	188.55±1.78	72.02	57.48	67.50
C.D. (p=0.05)			195.38	79.63			

Table 35: Effect of temperature on extraction of metals from bauxite ore by **Pseudomonas fluorescens**

Temperature	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
10^{0}C	2	2.176	88.34±0.47	72.33±0.32	12.13	22.05	15.12
15^{0}C	2	2.192	106.23±2.72	89.23±0.45	14.56	27.20	18.50
20^{0}C	2	2.208	122.41±2.92	106.60±1.43	16.81	32.50	21.68
24^{0}C	2	2.268	146.70±1.42	121.54±1.47	20.15	37.05	25.40
28^{0}C	2	2.323	192.30±1.47	162.46±1.92	26.41	49.39	33.59
32^{0}C	2	2.366	286.44±1.23	186.54±3.72	39.34	56.87	44.78
36^{0}C	2	2.448	278.21±1.56	176.54±1.04	38.21	53.82	43.06
40^{0}C	2	2.422	262.25±1.67	126.77±1.45	36.02	38.64	36.83
44^{0}C	2	2.396	256.00±1.47	120.00±1.44	35.16	36.58	35.60
48^{0}C	2	2.364	246.22±1.45	110.66±1.43	33.82	33.53	33.71
C.D. (p=0.05)			62.30	30.21			

Table 36: Effect of residence time on extraction of metals from bauxite ore by Thiobacillus ferrooxidans

Residence Time. (days)	Bauxite Ore in (g)		Metal extracted (mg)		Metal extracted %		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
4	2	2.264	167.66±1.06	86.34±1.02	23.03	26.32	24.05
8	2	2.297	190.34±1.04	107.87±1.86	26.14	32.88	28.23
12	2	2.523	295.00±2.10	124.54±1.06	40.52	37.96	39.72
16	2	2.556	311.34±2.13	146.78±1.08	42.76	44.75	43.38
20	2	2.594	364.56±1.76	178.37±1.23	50.07	54.38	51.41
24	2	2.623	378.89±1.76	186.43±1.56	52.04	56.83	53.53
28	2	2.678	403.67±2.65	221.64±1.02	55.44	67.57	59.21
32	2	2.764	523.45±2.34	246.25±1.34	71.9	75.07	72.88
36	2	2.868	565.87±1.23	268.51±1.52	77.72	81.86	79.01
40	2	2.892	606.67±2.92	286.23±1.34	83.33	87.26	84.55
C.D. (p=0.05)			121.24	56.28			

Table 37: Effect of residence time on extraction of metals from bauxite ore by **Pseudomonas fluorescens**

Residence Time. (days)	Bauxite ore (g)		Metal extracted (mg)		Metal extracted %		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
2	2	2.126	23.45 ± 0.07	12.33±0.27	3.22	3.75	3.38
4	2	2.188	70.43 ± 0.94	32.66±0.72	9.67	9.95	9.76
6	2	2.296	124.66 ± 0.98	63.33±0.76	17.12	19.3	17.8
8	2	2.324	178.56 ± 1.51	81.32±0.73	24.52	24.79	24.6
10	2	2.346	206.78 ± 0.98	108.00±0.47	28.4	32.92	29.8
12	2	2.392	241.34±0.98	132.66±0.76	33.15	40.44	35.41
14	2	2.436	260.66±0.72	163.43±0.98	35.8	49.82	40.16
16	2	2.484	274.45±0.94	186.00±0.94	37.69	56.7	43.6
18	2	2.496	282.00±0.47	218.23±0.47	38.73	66.53	47.34
20	2	2.523	291.33±0.98	236.34±0.47	40.01	72.05	49.96
C.D. (p=0.05)			76.59	62.69			

Table 38: Effect of pulp density on extraction of metals from bauxite ore by *Thiobacillus ferrooxidans*

Initial wt of bauxite ore (g)	Bauxite ore (g)	Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Final wt	Aluminum	Iron	Aluminum	Iron	
1	2.46	284.66±0.981	198.43±1.78	39.10	60.49	45.74
2	2.59	371.33±0.654	202.67±1.56	51.00	61.78	54.35
3	2.68	481.33±0.981	247.78±1.43	66.11	75.54	69.04
4	2.88	575.32±0.720	286.45±1.82	79.02	87.33	81.60
5	2.90	589.67±0.827	236.32±1.56	80.99	72.04	78.15
6	2.75	464.67±0.982	221.34±1.65	63.82	67.48	64.96
7	2.69	423.00±0.707	210.32±1.86	58.10	64.12	59.97
8	2.65	390.67±1.51	198.54±1.87	53.66	60.53	55.79
9	2.57	363.33±0.987	182.65±1.98	49.90	55.68	51.70
10	2.52	344.67±0.596	171.32±1.67	47.34	52.23	48.86
C.D. (p=0.05)		80.51	27.55			

Table 39: Effect of pulp density on extraction of metals from bauxite ore by **Pseudomonas fluorescens**

Initial wt of Bauxite ore (g)	Bauxite ore (g)	Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Final wt	Aluminum	Iron	Aluminum	Iron	
2	2.96	280.00±1.47	241.33±1.78	38.46	73.57	49.36
4	2.98	288.34±1.23	242.66±1.42	39.60	73.98	50.28
6	2.82	262.43±1.45	220.66±1.89	36.04	67.27	45.74
8	2.80	230.00±1.56	197.33±1.46	31.59	60.16	40.46

10	2.70	197.23±1.47	181.45±1.47	27.10	55.32	35.86
12	2.68	184.33±1.47	151.64±1.23	25.32	46.23	31.81
C.D. (p=0.05)		45.68	37.65			

Table 40: Effect of agitation on extraction of metals from bauxite ore by *Thiobacillus ferrooxidans*

Treatment Agitation In rpm	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
0	2	2.48	394.00±2.34	198.32±1.67	54.12	60.46	56.09
60	2	2.578	452.66±2.72	210.44±1.63	62.17	64.15	62.79
80	2	2.593	482.00±2.47	234.84±1.82	66.2	71.59	67.88
120	2	2.804	532.66±2.72	246.70±1.81	73.16	75.21	73.80
140	2	2.876	568.56±2.27	268.98±1.47	78.09	82	79.31
C.D. (p=0.05)			78.34	32.46			

Table 41: Effect of agitation on extraction of metals from bauxite ore by **Pseudomonas fluorescens**

Treatment Agitation In rpm	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
0	2	2.156	142.34±1.67	121.34±0.67	19.55	36.99	24.96
60	2	2.221	182.66±1.72	164.76±0.72	25.09	50.23	32.89
80	2	2.246	222.66±1.65	184.00±0.47	30.58	55.09	38.5
120	2	2.298	262.68±1.54	206.45±0.98	36.08	62.93	44.38
140	2	2.346	296.00±1.81	232.66±0.73	40.65	70.93	50
C.D. (p=0.05)			70.43	48.58			

Table 42: Effect of particle size on extraction of metals from bauxite ore by *Thiobacillus ferrooxidans*

Particle size In (mm).	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
0.20-0.40	2	2.864	585.33±2.78	260.00±1.47	80.4	79.26	80.05
0.40-0.80	2	2.843	574.32±3.43	245.00±1.48	78.89	74.69	77.58
0.80-1.20	2	2.808	559.33±1.13	229.33±1.72	76.83	69.91	74.68
1.20-2.00	2	2.796	541.66±2.36	218.34±1.36	74.4	66.56	71.96
2.00-2.80	2	2.767	527.82±2.23	202.45±2.36	72.5	61.72	69.15
2.80-4.00	2	2.723	516.33±1.36	180.87±2.49	70.92	55.14	66.02
4.00-5.00	2	2.692	492.66±1.72	165.23±1.59	67.66	50.37	62.3
5.00-6.00	2	2.686	482.66±2.72	144.00±1.84	66.29	43.9	59.34
C.D. (p=0.5%)			33.73	36.48			

Table 43: Effect of particle size on extraction of metals from bauxite ore by Pseudomonas fluorescens

Particle size In (mm).	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
0.20-0.40	2	2.542	266.05±1.81	236.78±1.94	36.53	72.18	47.61
0.40-0.80	2	2.532	252.45±1.47	210..45±1.88	34.67	64.16	43.83
0.80-1.20	2	2.521	243.33±1.36	195.33±2.72	33.42	59.55	41.53
1.20-2.00	2	2.508	228.66±1.27	180.34±2.27	31.4	54.98	38.73
2.00-2.80	2	2.496	212.66±1.27	162.79±1.47	29.21	49.63	35.55
2.80-4.00	2	2.474	192.68±1.81	135.43±1.72	26.37	41.28	31.07
4.00-5.00	2	2.443	181.66±1.36	121.56±1.36	24.95	37.06	28.71
5.00-6.00	2	2.432	164.23±1.98	111.33±1.75	22.55	35.46	26.56

Table 44: Effect of inoculum on extraction of metal from bauxite ore by *Thiobacillus ferrooxidans*

% Inoculum	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
10	2	2.623	439.20±1.84	142.34±1.63	60.30	43.39	55.05
20	2	2.674	465.32±1.59	156.68±1.47	63.91	47.76	58.90
30	2	2.689	478.67±2.27	164.22±1.23	65.75	50.06	60.87
40	2	2.71	485.78±1.75	172.45±2.47	66.72	52.57	62.33
50	2	2.764	497.46±1.36	180.13±2.72	68.33	54.91	64.16
60	2	2.787	510.40±2.47	198.44±3.42	70.00	60.50	67.08
70	2	2.823	526.66±2.62	221.86±2.72	72.34	67.64	70.88
80	2	2.856	544.00±3.98	241.48±1.04	74.72	73.62	74.38
90	2	2.878	570.60±1.20	258.96±1.92	78.29	78.95	78.50
100	2	2.986	592.00±2.94	268.83±1.72	81.31	81.96	81.51
C.D. (p=0.05)			38.78	36.39			

Table 45: Effect of inoculum on extraction of metals from bauxite ore by **Pseudomonas fluorescens**

Inoculum	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
10	2	2.176	121.40±1.42	112.00±1.37	16.67	34.14	22.10
20	2	2.234	136.22±1.92	118.77±1.47	18.71	36.21	24.14
30	2	2.266	156.23±1.72	142.33±1.43	21.46	43.39	28.27
40	2	2.298	178.40±1.82	152.11±1.47	24.50	46.37	31.29
50	2	2.326	196.63±2.72	172.23±2.62	27.00	52.50	34.92
60	2	2.368	224.21±2.47	186.00±2.72	30.79	56.70	38.84
70	2	2.396	262.20±2.34	196.66±1.20	36.01	59.95	43.45
80	2	2.446	284.45±1.23	210.33±1.45	39.07	64.12	46.85
90	2	2.462	298.43±1.40	214.44±1.43	40.99	65.37	48.56
100	2	2.587	326.60±1.08	221.31±1.28	44.86	67.47	51.88

C.D. (p=0.05)			58.43	32.29			

Table 46: Effect of NaCl concentration on extraction of metals from bauxite ore by *Thiobacillus ferrooxidans*

NaCl Con. (%)	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
0.50	2	2.987	586.00±2.47	288.23±2.43	80.49	87.87	82.76
1.00	2	2.978	581.66±2.45	278.43±2.67	79.89	84.88	81.44
1.50	2	2.964	575.33±2.75	266.60±2.78	79.02	81.28	79.67
2.00	2	2.794	482.87±3.34	232.45±2.23	66.32	70.86	67.73
2.50	2	2.723	421.66±2.67	216.34±1.84	57.92	65.95	60.41
3.00	2	2.689	385.67±1.76	206.67±2.65	52.97	63	56.08
3.50	2	2.584	332.87±1.32	192.00±1.73	45.72	58.53	49.7
4.00	2	2.496	284.67±2.34	178.52±2.45	39.1	54.42	43.86
4.50	2	2.378	204.88±2.54	162.34±1.65	28.14	49.49	34.77
5.00	2	2.223	165.00±1.56	132.64±1.68	22.43	40.43	28.16
C.D. (p=0.05)			126.18	41.83			

Table 47: Effect of NaCl concentration on extraction of metals from bauxite ore by **Pseudomonas fluorescens**

NaCl Con. (%)	Bauxite ore (g)		Metal extracted (mg)		Metal extracted %		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
0.50	2	2.71	283.33±2.72	178.43±1.89	38.91	54.39	43.72
1.00	2	2.698	268.45±2.94	162.65±1.83	36.87	49.58	40.41
1.50	2	2.682	242.66±2.78	146.00±1.34	33.33	44.51	36.80
2.00	2	2.623	228.68±1.67	138.43±1.56	31.41	42.20	34.76
2.50	2	2.593	193.33±1.98	123.34±1.68	26.55	37.60	29.98
3.00	2	2.578	181.23±1.62	117.45±1.65	24.89	35.8	28.28
3.50	2	2.542	164.43±2.78	110.67±1.56	22.58	33.74	26.05
4.00	2	2.486	143.89±1.94	98.45±1.43	19.76	30.01	22.94
4.50	2	2.38	108.34±1.94	87.67±1.60	14.88	26.72	18.56
5.00	2	2.187	82.00±1.76	72.42±0.48	11.26	22.07	14.58
C.D. (p=0.05)			54.64	27.09			

Table 48: Comparative effect of *Thiobacillus ferrooxidans* cells and cell free extract on metal extraction

Treatment	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
With *Thiobacillus ferrooxidans*	2	2.964	545.33±2.72	268.43±1.24	74.9	81.83	77.06
Without	2	2.21	262.23±2.59	184.64±1.78	36.02	56.29	43.44

Thiobacillus ferrooxidans							
Control	2	1.998	4.88±0.68	6.22±0.64	0.67	1.89	1.05
C.D. (p=0.05)			141.55	107.67			

Table 49: Comparative effect of ***Pseudomonas fluorescens*** cells and cell free extract on metal extraction

Treatment	**Bauxite ore (g)**		**Metal extracted (mg)**		**Metal extracted (%)**		**Bioextraction Efficiency of Al and Fe (%)**
	Initial wt	**Final wt**	**Aluminum**	**Iron**	**Aluminum**	**Iron**	
With **Pseudomonas fluorescens**	2	2.223	134.64±1.08	188.23±1.23	18.49	57.38	30.57
Without **Pseudomonas fluorescens**	2	1.991	96.94±1.54	72.14±1.47	13.31	21.99	16.01
Control	2	1.998	4.88±0.86	6.22±0.76	0.67	1.89	1.05
C.D. (p=0.05)			48.44	149.18			

Table 50: Synergistic effect of *Thiobacillus ferrooxidans* and other bacteria on extraction of metals from bauxite ore

Thiobacillus + other microbes	**Bauxite ore (g)**		**Metal extracted (mg)**		**Metal extracted (%)**		**Bioextraction Efficiency of Al and Fe (%)**
	Initial wt	**Final wt**	**Aluminum**	**Iron**	**Aluminum**	**Iron**	
Thiobacillus ferrooxidans+ **Pseudomonas fluorescens**	2	2.868	464.32±2.95	266.45±1.27	63.68	81.23	69.2
Thiobacillus ferrooxidans+ Pseudomonas aeruginosa	2	2.723	381.00±2.84	268.56±1.08	52.33	81.87	61.51
Thiobacillus ferrooxidans+ Bacillus megaterium	2	2.72	447.67 ± 1.76	287.43±1.02	61.49	87.63	69.61
Thiobacillus ferrooxidans+ Bacillus polymyxa	2	2.706	431.32 ± 2.98	236.78±1.10	59.24	72.18	63.26
Thiobacillus ferrooxidans+ Bacillus cereus	2	2.684	395.43 ± 2.84	240.23±1.23	54.31	73.24	60.19
C.D. (p=0.05)			132.37	60.57			

Table 51: Synergistic effect of ***Pseudomonas fluorescens*** and other bacteria and on extraction of metals from bauxite ore

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Pseudomonas+ microbes	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
Pseudomonas fluorescens Pseudomonas aeruginosa	2	2.564	318.45±2.94	255.33±1.196	43.74	77.84	54.33
Pseudomonas Pseudomonas cissicola	2	2.348	268.67±2.72	239.33±2.78	36.9	72.96	48.10
Pseudomonas Bacillus polymyxa	2	2.334	242.78±2.78	191.45±2.98	33.34	58.36	41.12
Pseudomonas Bacillus megaterium	2	2.278	218.43±1.54	182.66±2.72	30	55.58	37.98
Pseudomonas Bacillus megaterium	2	2.347	246.23±1.16	227.56±2.84	33.82	69.37	44.86
Pseudomonas fluorescens	2	2.321	244.00±1.69	169.00±2.84	33.51	51.52	39.10
C.D. (p=0.05)			36.00	36.32			

Table 52: Synergistic effect of fungi and *Thiobacillus ferrooxidans* on extraction of metals from bauxite ore

Thiobacillus ferrooxidans **+**fungi	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
Thiobacillus+Aspergillus niger	2	2.846	364.32±2.49	196.45±1.78	50.04	59.89	53.10
Thiobacillus+Aspergillus flavus.	2	2.806	342.00±2.94	176.60±1.85	46.97	53.84	49.10
Thiobacillus +Fusarium oxysphorum.	2	2.782	323.00±2.70	164.23±1.45	44.36	50.07	46.13
Thiobacillus +Penicillium sp.	2	2.796	332.00±2.94	172.43±1.34	45.60	52.57	47.76
Thiobacillus+ Rhizopus arrihizus.	2	2.852	365.00±2.70	168.67±1.56	50.13	51.42	50.53
Thiobacillus ferrooxidans	2	2.872	568.34±2.87	276.56±1.65	78.06	84.31	79.96
C.D. (p=0.05)			97.19	44.77			

Table 53: Synergistic effect of fungi and ***Pseudomonas fluorescens*** on extraction of metals from bauxite ore

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***Pseudomonas fluorescens* +** Fungi	**Bauxite ore (g)**		**Metal extracted (mg)**		**Metal extracted (%)**		**Bioextraction Efficiency of Al and Fe (%)**
	Initial wt	**Final wt**	**Aluminum**	**Iron**	**Aluminum**	**Iron**	
Pseudomonas Aspergillus niger	2	3.624	247.33±1.27	180.66±2.78	33.97	55.07	40.52
Pseudomonas Aspergillus flavus	2	3.384	168.00±2.16	120.43±1.72	23.07	36.71	27.31
Pseudomonas Penicillium sp.A	2	3.487	220.43±1.59	191.33±2.27	30.27	58.33	38.99
Pseudomonas Penicillium sp.	2	3.227	202.66±1.18	184.33±1.59	27.83	56.19	36.64
Pseudomonas Fusarium sp.	2	3.110	182.66±0.76	92.00±0.94	25.09	28.04	26.00
Pseudomonas fluorescens	2	2.976	263.56±1.98	198.67±1.75	36.20	60.57	43.77
C.D. (p=0.05)			38.79	46.16			

Table: 54 Quantitative analysis of fungal species occurrence in fly ash.

Fungal species	**Fungal population in (%)**
Aspergillus niger	18.42
Aspergillus fumigatus	15.46
Penicillium sp.	15.6
Aspergillus flavus	12.1
Penicillium notatum	13.63
Aspergillus terreus	10.08
Fusarium sp.	9.47
Aspergillus carboniferous	4.54
Helminthosporium sp.	3.48
Bacteria c.f.u./g	$21x\ 10^2$
Actinomycetes c.f.u./g	$8x10^2$

Table: 55 Extraction of metals from fly ash by adapted and non-adapted strains of *Thiobacillus ferrooxidans*.

Treatment	**Metal solublization in media (mg/g)**			**Bioextraction of metals (%)**			**Bioextraction Efficiency (%)**
	Al	**Fe**	**Mn**	**Al**	**Fe**	**Mg**	
Non-adapted *Thiobacillus ferrooxidans*	196.66±2.34	82.21±2.84	10.25±1.64	79.94	87.47	72.85	81.65
Adapted *Thiobacillus ferrooxidans.*	116.42±1.45	64.22±1.94	7.85± 2.45	66.02	68.31	56.07	66.24
control	4.22±1.34	2.44± 2.3	0.84±1.60	1.62	2.59	6	7.5
C.D. (p=0.05)	103.11	23.12	3.08				

Table: 56 Extraction of metals from fly ash by adapted and non-adapted strains of **Pseudomonas fluorescens**.

Treatment	Metal solublization in media (mg/g)			Bioextraction of metals (%)			Bioextraction Efficiency (%)
	Al	Fe	Mn	Al	Fe	Mg	
Non-adapted **Pseudomonas fluorescens**.	36.68 ±1.43	42.88± 2.46	1.32±0 .22	14.91	45.61	9.42	22.84
Adapted **Pseudomonas fluorescens**.	40.84 ±1.86	46.30± 1.89	2.84±1 .24	16.60	49.25	20.28	25.41
Control	2.44± 1.08	3.96±1 .66	0.42±0 .2	0.99	4.21	3.00	1.92
C.D. (p=0.05)	5..35	4..39	1. 95				

Table: 57 Effect of pH on extraction of metals from fly ash by *Thiobacillus ferrooxidans*.

pH	Metal solublization in media (mg/g)			Bioextraction of metals (%)			Bioextraction Efficiency (%)
	Al	Fe	Mn	Al	Fe	Mg	
1.0	168.22± 2.44	76.88± 2.48	6.96± 1.24	68.38	81.78	49.28	71.18
1.5	186.48± 2.00	80.44± 2.23	7.82± 1.44	75.80	85.57	55.71	75.47
2.0	208.70± 1.44	78.21± 4.22	10.0± 1.28	84.83	83.20	71.42	81.56
2.5	196.28± 2.66	82.76±1.22	9.84± 1.64	79.78	88.04	70.00	81.54
3.0	178.20± 3.66	76.24±1.44	8.22± 1.86	72.43	81.10	58.57	74.19
4.0	165.83± 2.87	72.10±1.60	6.54± 1.46	67.41	76.70	46.42	69.04
5.0	142.60± 4.22	64.20± 2.00	6.23± 1.44	57.96	68.29	44.28	60.16
6.0	126.28± 2.45	62.98±2.34	5.44± 1.78	51.33	67.00	38.57	54.97
7.0	112.96± 1.86	46.34±1.34	5.22± 1.88	45.91	49.29	37.14	46.48
8.0	92.46± 2.44	41.14±1.78	5.00± 1.85	37.58	43.76	35.71	39.15
C.D. (p=0.05)	30.87	11.72	1.47				

Table: 58 Effect of pH on extraction of metals from fly ash by **Pseudomonas fluorescens**.

pH	Metal solublization in media (mg/g)			Bioextraction of metals (%)			Bioextraction Efficiency (%)
	Al	Fe	Mn	Al	Fe	Mg	
5.0	14.66 ± 0.68	26.08 ± 0.46	1.06 ± 2.40	5.95	27.65	7.57	11.78
6.0	20.96 ± 2.44	32.44 ± 2.46	1.06 ± 1.22	8.52	34.51	14.00	15.63
7.0	26.46 ± 2.22	38.84 ± 1.44	1.96 ± 1.49	10.75	41.31	17.57	19.14
8.0	30.22 ± 1.46	40.86 ± 1.34	2.46 ± 2.00	12.28	43.46	8.64	20.42
9.0	21.38 ± 2.44	22.76 ± 0.68	1.21 ± 2.43	8.69	24.21	7.02	12.74

10.0	16.32 ± 1.48	18.88 ± 0.40	0.98 ± 1.24	6.59	20.08	6.14	10.15
Control	4.66 ± 1.33	3.38 ± 1.88	0.86 ± 1.88	1.89	3.59	4.57	2.45
C.D. (p=0.05)	8.15	12.54	0.58				

Table: 59 Effect of temperature on extraction of metals from fly ash by *Thiobacillus ferrooxidans*.

Temperature ^{0}C	**Metal solublization in media (mg/g)**			**Bioextraction of metals (%)**			**Bioextraction Efficiency (%)**
	Al	**Fe**	**Mn**	**Al**	**Fe**	**Mg**	
10 ^{0}C	64.20± 1.22	24.48±1.42	2.44± 0.86	26.09	26.04	17.42	25.74
20 ^{0}C	76.48± 1.64	26.58±1.21	4.64± 1.42	31.08	28.27	33.28	30.42
25 ^{0}C	82.90± 2.46	34.22±1.30	5.23± 1.88	33.69	36.4	37.35	34.56
28 ^{0}C	96.45± 2.44	40.10±2.43	7.04± 0.96	39.2	42.65	50.28	40.54
32 ^{0}C	142.32± 2.7	46.20±2.00	8.50± 1.65	57.85	49.14	60.71	55.65
35 ^{0}C	182.66± 4.22	76.80±2.67	10.2± 1.44	74.25	81.7	73	76.18
38 ^{0}C	186.24± 4.64	84.50±2.54	10.4± 1.34	75.7	89.93	74.57	79.44
40 ^{0}C	172.68± 1.34	86.42±2.48	9.68± 1.74	70.19	91.93	69.14	75.92
42 ^{0}C	164.82± 1.44	78.22±1.45	9.21± 1.62	67.24	83.21	65.78	71.25
45 ^{0}C	134.44± 1.68	68.23±1.65	8.44± 1.55	54.65	72.58	60.28	59.63
C.D. (p=0.05)	38.03	20.08	2.17				

Table: 60 Effect of temperature on extraction of metals from fly ash by **Pseudomonas fluorescens**.

Temp.	**Metal solublization in media (mg/g)**			**Bioextraction of metals (%)**			**Bioextraction Efficiency (%)**
	Al	**Fe**	**Mn**	**Al**	**Fe**	**Mg**	
10 ^{0} C	6.83 ± 1.45	14.22 ± 3.45	0.32 ± 0.4	2.77	15.12	2.28	6.03
20 ^{0} C	22.46 ± 2.44	26.44 ± 2.42	0.86 ± 0.20	9.13	28.12	6.14	14.05
25 ^{0} C	26.96 ± 4.68	36.23 ± 1.88	1.20 ± 0.34	10.95	38.54	8.57	18.34
30 ^{0} C	36.04 ± 2.43	53.96 ± 1.54	1.96 ± 0.46	14.65	57.40	14.00	25.97
34 ^{0} C	40.21 ± 1.65	60.12 ± 1.44	2.86 ± 0.24	16.34	63.95	20.42	29.14
38 ^{0} C	37.48 ± 0.66	48.22 ± 1.78	1.48 ± 0.66	15.23	51.29	10.57	24.62
42 ^{0} C	31.28 ± 1.22	52.33 ± 2.45	2.40 ± 0.28	12.71	55.67	17.14	24.29
44 ^{0} C	26.84 ± 1.44	54.44 ± 3.40	2.10 ± 0.34	10.91	57.91	15.00	23.55
46 ^{0} C	21.46 ± 0.43	46.23 ± 4.22	1.98 ± 0.26	8.72	49.18	14.16	19.68
48 ^{0} C	18.22 ± 0.64	42.81 ± 1.43	1.10 ± 0.44	7.40	45.54	7.85	17.55
C.D. (p=0.05)	8.22	11.54	0.63				

Table: 61 Effect of residence time on extraction of metals from fly ash by *Thiobacillus ferrooxidans*.

Time (days)	**Metal solublization in media (mg/g)**			**Bioextraction of metals (%)**			**Bioextraction Efficiency (%)**
	Al	**Fe**	**Mn**	**Al**	**Fe**	**Mg**	
2	9.22± 0.84	2.20± 2.68	1.22± 0.96	3.74	2.34	8.71	3.56
6	16.48±1.46	8.42± 2.36	3.40± 2.34	6.69	8.95	24.28	7.99
10	64.42± 2.44	24.84±2.56	6.82± 1.48	26.18	26.42	48.71	27.14
14	86.34± 5.66	62.21±1.22	8.66± 1.89	35.09	66.18	61.85	44.4

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20	126.92±2.86	74.30±1.78	9.22± 2.33	51.59	79.04	65.85	59.44
24	146.28±1.23	82.12±2.44	10.44± 1.6	60.46	87.36	74.57	67.46
C.D. (p=0.05)	58.92	36.54	3.86				

Table: 62 Effect of residence time on extraction of metals from fly ash by **Pseudomonas fluorescens**.

Time (days)	Metal solublization in media (mg/g)			Bioextraction of metals (%)			Bioextraction Efficiency (%)
	Al	Fe	Mn	Al	Fe	Mg	
2	4.24 ± 1.88	6.25 ± 2.46	0.82 ± 0.22	1.72	6.64	5.85	3.19
4	10.84 ± 0.28	18.48 ± 2.00	0.98 ± 0.42	4.40	19.65	7.00	8.56
6	18.84 ± 2.56	26.90 ± 1.22	1.22 ± 0.82	7.51	28.61	8.71	13.18
8	26.78 ± 2.66	36.84 ± 4.66	1.96 ± 0.98	10.88	39.19	14.00	18.52
10	30.68 ± 1.33	42.44 ± 4.80	2.40 ± 0.68	12.47	45.14	17.14	21.33
12	32.96 ± 1.22	56.77 ± 2.68	2.98 ± 1.22	13.39	60.39	21.28	26.18
Control	4.00 ± 0.84	3.25 ± 1.23	0.42 ± 0.21	1.62	3.45	3.00	2.16
C.D. (p=0.05)	11.88	18.97	0. 90				

Table: 63 Effect of substrate concentrations on extraction of metals from fly ash by *Thiobacillus ferrooxidans*.

Initial wt of fly ash in (g)	Metal solublization in media (mg/g)			Bioextraction of metals (%)			Bioextraction Efficiency (%)
	Al	Fe	Mn	Al	Fe	Mg	
2	86.23±1.33	39.42±0.32	6.78±2.4	71.66	81.25	77.56	74.54
4	124.20±1.6	74.86±1.86	8.5±1.32	50.4	78.72	60.71	58.33
6	164.0±0.60	104.65±2.4	16.88±1.6	45.05	72.22	66.68	53.38
8	114.22±2.0	68.96±1.45	18.44±1.8	34.16	34.69	56.25	28.24
10	104.45±2.2	44.56±1.44	10.22±1.4	17.21	18.33	23.8	17.83
12	96.00±1.46	42.22±2.45	8.46±2.46	13.25	14.68	14.81	13.72
Cont-	4.2±1.34	2.34±0.45	0.66±1.23	2.39	4.83	6.74	4.071
C.D. (p=0.05)	28.84	26.70	5.12				

Table: 64 Effect of substrate concentrations on extraction of metals from fly ash by **Pseudomonas fluorescens**.

Initial wt. of fly ash (g)	Metal solublization in media (mg/g)			Bioextraction of metals (%)			Bioextraction Efficiency (%)
	Al	Fe	Mn	Al	Fe	Mg	
2	26.18±1.22	41.23±0.86	1.22±0.44	11.07	85.89	15.25	38.99
4	36.48±2.43	46.20±1.84	2.46±0.84	14.82	49.14	17.50	24.04
6	40.30±2.45	52.00±1.23	2.64±0.22	21.81	36.11	11.00	17.84
8	32.22±1.45	50.24±1.66	1.26±1.42	6.71	25.63	3.93	11.82
10	26.40±1.68	42.22±2.45	0.86±1.34	4.37	17.59	2.04	7.84
12	20.80±1.26	36.10±2.40	0.76±1.88	1.95	12.62	1.40	5.41
Con	4.26±1.45	4.89±1.56	0.86±1.68	1.73	5.14	6.14	2.82
C.D. (p=0.05)	7.63	6.27	0.85				

Table 65: Extraction of metals from bauxite ore by cured and non-cured *Thiobacillus ferrooxidans*

Treatment	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
Acridine orange Treated *Thiobacillus ferrooxidans*	2	2.678	536.24 ±1.23	268.78 ± 1.10	73.65	81.94	76.23
Ethium bromide Treated *Thiobacillus ferrooxidans*	2	2.663	526.23 ±1.08	278.00 ± 1.04	72.28	84.75	76.15
Thiobacillus ferrooxidans	2	2.685	542.66 ±2.68	286.68 ± 2.98	74.54	87.4	78.53
C.D. (p=0.05)			12.29	13.28			

Table 66: Extraction of metals from bauxite ore by cured and non- cured Pseudomonas fluroscens

Treatment	Bauxite ore (g)		Metal extracted (mg)		Metal extracted (%)		Bioextraction Efficiency of Al and Fe (%)
	Initial wt	Final wt	Aluminum	Iron	Aluminum	Iron	
Acridine orange Treated **Pseudomonas fluorescens**	2	2.433	196.84 ± 2.78	186.48 ± 1.48	27.03	56.85	36.29
Ethium bromide Treated **Pseudomonas fluorescens**	2	2.487	182.23 ± 2.34	168.00 ± 1.42	25.03	51.21	33.16
Pseudomonas fluorescens	2	2.524	208.66 ± 2.44	195.33 ± 1.78	28.66	59.55	38.25
C.D. (p=0.05)			19.64	20.69			

Table: 67 Number of amplified fragments of Thiobacillus and Pseudomonas mutants based on ISSR analysis using four primers

Primers used	TAF	PB	PB (%)	MB	MB (%)	Ratio of PB and MB
UBC-861	17	05	29.41	12	70.58	1: 2.39
UBC-862	14	06	42.85	08	57.14	1: 1.33
UBC-867	18	06	33.33	12	66.66	1: 2.00
UBC-868	08	00	00.00	08	100	1: 0.00
Total	57	17	29.82	40	70.17	1: 2.35

Table: 68 List of ISSR primers used for PCR amplification.

Sr.no.	Primer code	Primer sequence

1	UBC-811	GAG AGA GAG AGA GAG AC
2	UBC-812	GAG AGA GAG AGA GAG AA
3	UBC-813	CTC TCT CTC TCT CTC TT
4	UBC-814	CTC TCT CTC TCT CTC TA
5	UBC-861	ACC ACC ACC ACC ACC
6	UBC-862	AGC AGC AGC AGC AGC AGC
7	UBC-867	GGC GGC GGC GGC GGC GGC
8	UBC-868	GAA GAA GAA GAA GAA GAA

Among the eight primer tested four proved useful to characterize this samples table 67 where as four were excluded due to absence of amplification.

Table: 69 Amplified fragments (base pair) of primer No.861 Primer sequence 5'ACC ACC ACC ACC ACC ACC 3'

Sample name	**T. ferrooxidans,**	**T. ferrooxidans (UV 26)**	**P. fluorescens**	**P. fluorescens (ISO 24)**
Lane	1	2	3	4
Fragment size In bp	-	-	-	975
	940	-	-	
	800	800	800	800
	775	775	775	775
	-	-	-	760
	740	-	-	-
	-	-	700	-
	645	645	645	645

Table: 70 Amplified fragments (base pair) of primer No.862 Primer sequence 5'AGC AGC AGC AGC AGC AGC 3'

Sample name	**T. ferrooxidans,**	**T. ferrooxidans (UV 26)**	**P. fluorescens**	**P. fluorescens (ISO 24)**
Lane	1	2	3	4
Fragment size In bp	1510	1510	1510	1510
	-	-	1290	-
	700	700	700	700
	-	490	490	490
	-	-	-	410
	-	-	345	-

Table: 71 Amplified fragments (base pair) of primer No.867

Sample Name	***Thiobacillus ferrooxidans*,**	***Thiobacillus ferrooxidans* (UV 26)**	**Pseudomonas fluorescens**	**Pseudomonas fluorescens (ISO 24)**
Lane	**1**	**2**	**3**	**4**
Fragment size In bp	-	-	1860	-
	1210	1210	1210	1210
	-	900	-	-
	-	620	-	-
	600	600	600	600
	-	510	-	-
	480	480	480	480
	270	270	-	-

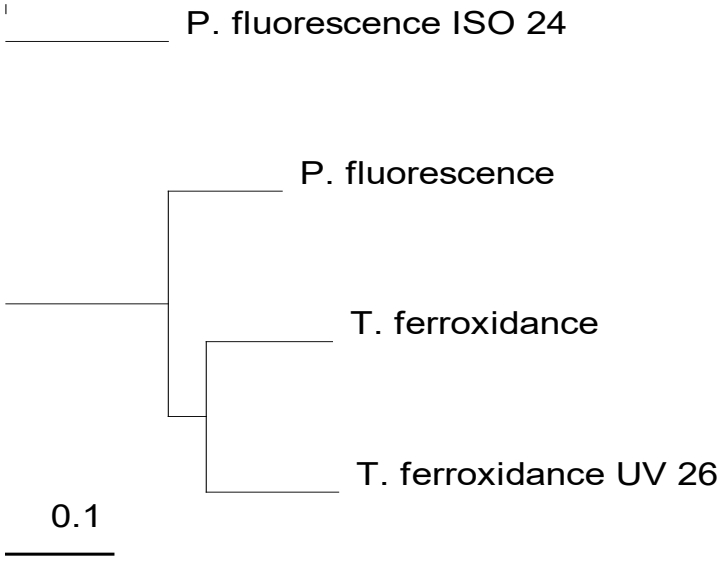
Primer sequence 5'GGC GGC GGC GGC GGC GGC 3'

Table: 72 Amplified fragments (base pair) of primer No.868 Primer sequence 5'GAA GAA GAA GAA GAA GAA 3'

Sample name	**Thiobacillus ferrooxidans**	***Thiobacillus ferrooxidans* (UV 26)**	**Pseudomonas fluorescens**	**Pseudomonas fluorescens (ISO 24)**
Lane	**1**	**2**	**3**	**4**
Fragment size In bp	530	530	530	530
	370	370	370	370

Dendrogram

The Dendrogram analysis was carried out by using bioinformatics phylogeny tool Free Tree and Tree View of DNA fingerprint analysis. The dendrogram tree was mentioned below.



Phylogenetic Analysis

Distance Similarity Matrix:

Table: 73 Distance Similarity Matrix was calculated by using Neighbor-joining tree construction method of Nei and Li (1979) Dice index.

	Thiobacillus ferrooxidans	**Thiobacillus ferrooxidans (UV 26)**	**Pseudomonas fluorescens**	**Pseudomonas fluorescence (ISO 24)**
Thiobacillus ferrooxidans		0.24138	0.25926	0.30769
Thiobacillus Ferrooxidans (UV 26)	0.24138		0.26667	0.31034
Pseudomonas fluorescens	0.25926	0.26667		0.25926
Pseudomonas fluorescens (ISO 24)	0.307690222	0.31034	0.25926	

Table 74: Molecular weight (KDa) of polypeptide bands of *Thiobacillus ferrooxidans* interacted with metal ions (plate V-A)

Lan-1	Lan-2	Lan-3	Lan-4	Lan-5	Lan-6	Lan-7	Lan-8	Lan-9
120.5	118.5	119.2	114.0	117.2	120.2	118.2	121.0	118.8
94.8	92.4	90.2	98.2	96.2	94.2	98.4	92.4	91.6
83.4	81.6	78.2	76.4	77.8	82.4	90.2	81.2	82.8
74.8	72.4	68.4	64.3	71.2	72.8	76.4	72.6	76.2
66.0	65.2	63.4	60.2	64.8	65.4	70.5	64.2	68.4
48.2	48.2	42.4	40.2	46.7	49.7	68.1	46.7	46.1
45.4	40.3	40.2	38.4	44.2	47.4	49.4	42.1	42.6
43.8	43.4	42.4	32.2	41.2	45.2	46.2	39.8	40.2
29.5	28.4	27.5	26.5	29.0	42.9	36.7	26.7	32.4
15.2	14.2	14.6	14.4	15.6	36.2	26.8	16.8	16.8
15.0	14.5	14.9	12.4	15.2	30.4	18.3	14.6	14.2
14.3	13.5	13.0	12.0	14.2	26.5	15.5	14.0	14.0
12.6	12.2	12.0	11.2	12.4	18.8	14.4	12.8	12.8
10.2	10.2	10.6	10.2	10.2	14.4	12.6	10.9	10.4
10.0	10.0	10.5	10.0	10.6	12.2	10.8	10.4	10.0

Table 75: Molecular weight (KDa) of polypeptide bands of ***Pseudomonas fluorescens*** interacted with metal ions (plate V-A)

sLan-1	Lan-2	Lan-3	Lan-4	Lan-5	Lan-6	Lan-7	Lan-8	Lan-9
118.5	116.5	114.2	114.0	117.2	114.5	116.4	118.6	116.0
96.8	92.4	94.2	96.2	96.2	92.4	98.2	90.2	94.6
83.4	81.6	78.2	76.4	77.8	82.2	79.8	86.2	80.8
74.8	71.4	68.4	64.3	71.2	71.2	71.2	71.4	76.4
66.0	64.2	62.4	60.2	64.8	68.4	68.2	65.4	66.2
44.2	48.2	42.4	40.2	46.7	42.6	49.5	41.2	41.4
45.4	40.3	40.2	38.4	44.2	40.2	47.6	46.4	38.4
43.8	43.4	42.4	32.2	41.2	36.2	41.2	40.8	36.4
22.5	28.4	27.5	26.5	29.0	24.8	26.8	29.8	21.4
15.2	14.2	14.6	14.4	15.6	14.8	16.4	21.4	16.8
15.0	14.5	14.9	12.4	15.2	14.0	15.8	16.5	15.9
14.3	13.5	13.0	12.0	14.2	12.6	14.8	15.6	14.6
12.6	12.2	12.0	11.2	12.4	12.0	14.0	14.3	12.5
10.2	10.2	10.6	10.2	10.2	10.8	12.8	12.8	12.0
10.0	10.0	10.5	10.0	10.6	10.2	10.9	10.9	10.5

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ABOUT THE BOOK

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