IRON BIOPRECIPITATION AND REMEDIATION OF METALLIC POLLUTANTS

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UNDER THE GUIDANCE OF

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Certificate

*This is to certify that the work presented in this thesis entitled "**Iron bioprecipitation and remediation of metallic pollutants**" submitted by **Rajput Rashmi** has been carried out under my supervision in the Department of Microbiology, School of Sciences, Gujarat University, Ahmedabad, for the Degree of Doctor of Philosophy in Biotechnology.*

I, further, testify that this thesis or part thereof has not previously been formed the basis for the award of any degree, diploma, associateship, fellowship or other similar awards.

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*I, **Ms Rajput Rashmi**, the undersigned hereby solemnly declare that the work presented in the thesis entitled **"Iron bioprecipitation and remediation of metallic pollutants"** is original. I declare further that this work has not been submitted for any degree or diploma to any other University or institution.*

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"Gratitude is the fairest blossom, which spring from the soul"

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ABBREVIATIONS

FAC	Ferric Ammonium Citrate
CB	Citrate Broth
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
CGY	Casitone Glycerol Yeast Autolysate Broth
rpm	Revolution Per Minute
ppm	Parts Per Million (mg/L)
TDS	Total Dissolved Solids
ORP	Oxidation Reduction Potential
°C	Degree Celsius/Centigrade
OD	Optical Density
g/L	Gram Per Litre
rDNA	Ribosomal Deoxy Nucleic Acid
sp.	Species
Se (VI)	Selenate
Se (IV)	Selenite
PYG-Mn	Peptone Yeast Glucose Manganese
ml	Millilitre
mM	Mill molar
NB	Nutrient Broth
TSC	Tri- Sodium Citrate
TAC	Tri- Ammonium Citrate

1 Chapter

Review of Literature

Environment has been defined as the "Sum total of all the conditions and influence that affect the development of the life of organisms". Environment performs main three functions:

1. It provides living space.
2. It provides resources such as air, water, minerals and soil.
3. It acts as a sink by assimilating the waste produced by human (Bhattacharya and Banerjee, 2007)

Environment consists of physicochemical surrounding and all the living things in the vicinity (Sondge, 2007).

The word "PARYAVARAN" is derived from compound word "PARI"+ "AAVARAN". The prefix "Pari" means surroundings and the suffix "Aavaran" means cover. In simple words PARYAVARAN means the natural surroundings, which include coverage of air, water, earth and sky where we live and move. The comprehensive definition of environment is the entire material and biological state and arrangement, in which all the creatures take birth and develop naturally with their own instincts. Moreover, the Vedic literature regard that ecology is compose of five spirits - Panchamahabhuts called Air, Water, Earth, Sky and Fire, but at present only three elements is included in ecology viz. Air, Water and Earth. The environment affects our physical, mental and spiritual health and therefore clean, clear and unpolluted environment is needed. The air we breathe gives oxygen, the water we drink that quenches our thirst, the earth (soil) that gives food, feeding and developing our physic must be pure and clean (Deshpande, 2007).

There are many polluting substances that cause disruption or change in the chemical makeup of the world's water and affect the aquatic environment. Some basic pollutants include radioactive material, sediments, inorganic chemicals, oil spills, synthetic organic compounds and toxic metals (www.umich.edug/gs265).

Environmental pollution is now considered as global phenomena. Pollution is the introduction by man into the environment a substance or energy liable to cause hazards to human health, harm to living resources and ecological systems, damage to structures or amenity or interference with legitimate uses of the environment (Atodaria, 2001). Different sources of pollution are shown in Figure 1 and Environmental pollution in Figure 2.

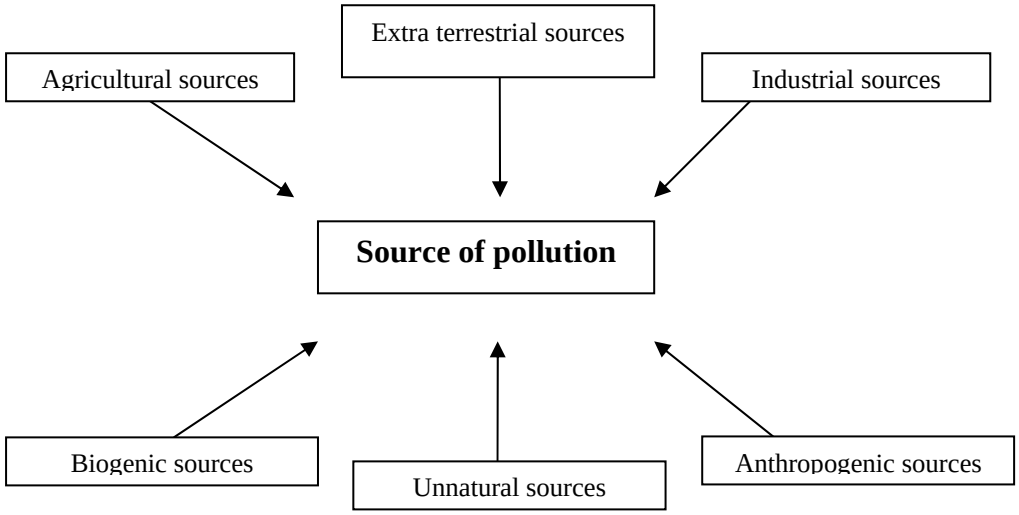


Figure1. Different sources of pollution (Santra, 2005)

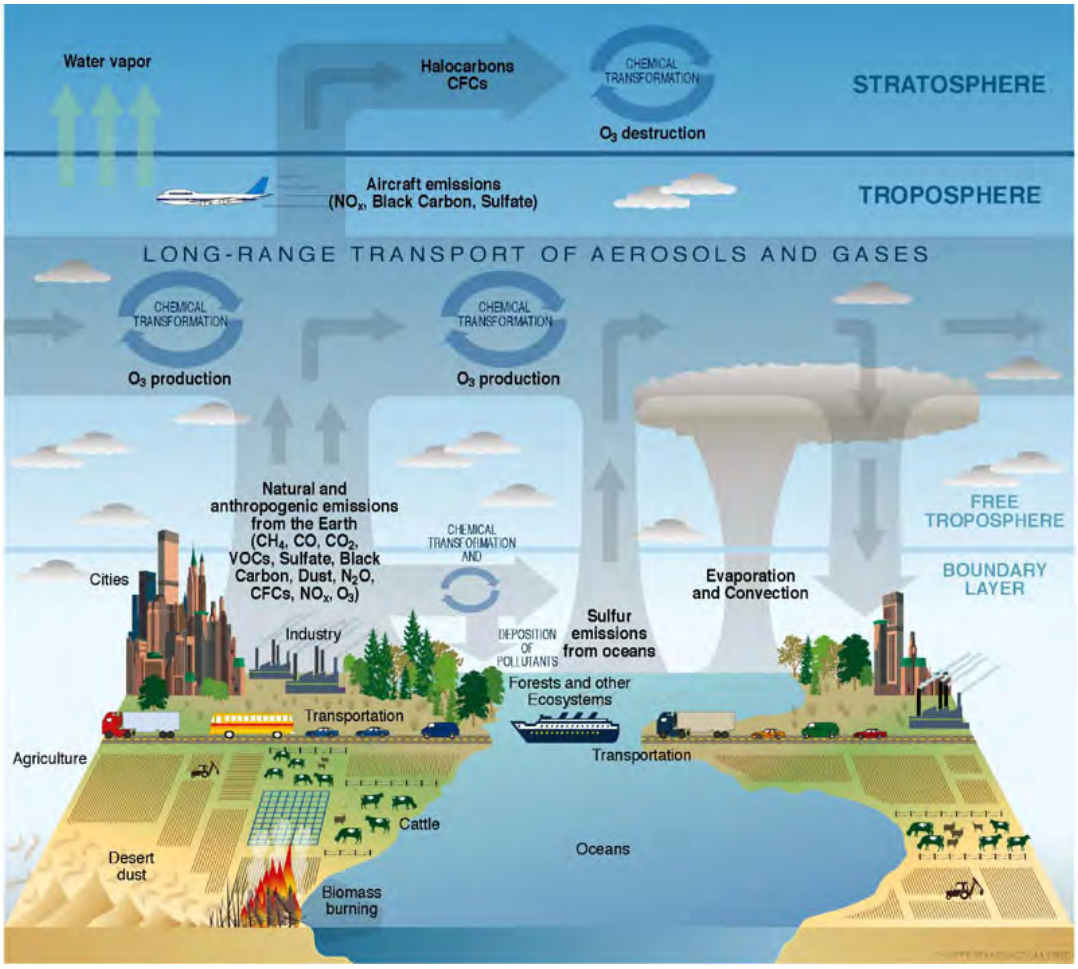


Figure 2. Environmental pollution

http://oceanworld.tamu.edu/resources/oceanography-book/Images/Atmosphere_composition_diagram.jpg

The major sources of pollution are waste disposal sites, scrap yards, gas works, petroleum refineries, coal mines, coal storage, electricity generation, iron and steel works, metalliferrous mining, smelting, chemical works, glass making, ceramic industries, textile plants, dye works, leather tanneries, timber products, semiconductors manufacturing, food processing, water treatment works, sewage works, asbestos works, paper manufacturing, printing works, heavy

engineering installation and radioactive waste processing (Atodaria, 2001).

Types of environmental pollution

There are three forms of environmental pollutants namely gases, liquid and solids. Waste discharge from various sources pollutes water, which leads to pollution in terms of physical pollution, chemical pollution, physiological and biological pollution (Bhattacharya and Banerjee, 2007).

1. **Physical pollution**

   Physical pollution of water is caused by the solid constituents of industrial effluents and sewage water. The nature of these solids varies depending on the type of industries. For eg. tannery effluent contains calcium carbonate, hair, flesh etc (Bhattacharya and Banerjee, 2007).

2. **Chemical pollution**

   The widespread use of chemicals in agriculture and industries without the availability of proper toxicological information on the chemicals has multiplied the hazards, to which human beings are exposed. Examples are acids, salts and alkalis, pesticides, fertilizer and petroleum hydrocarbon (Dhameja, 2004).

3. **Physiological pollution**

   It is also caused by the soluble chemicals and colloidal substances present in waste water. After the removal of suspended particles, the effluent can become harmless in general sense. For eg. H_2S is harmless at a concentration lower than 15 ppm but can be smelled even at a concentration of 0.001 ppm but this concentration is not

harmful, it can be tested even at such a low concentration (Bhattacharya and Banerjee, 2007).

4. Biological pollution

It is caused by the organic compound present in waste water or solid wastes. The various types of microorganisms present in air, water and soil decompose these polymeric complex compounds into carbon dioxide and water by consuming large quantities of dissolved oxygen. There by rendering the water or surrounding oxygen deficient (Bhattacharya and Banerjee, 2007). Various environmental pollutants are listed in Table 1.

Table 1. Environmental Pollutants (Scragg, 2005**)**

Types of pollutants	Example
Inorganic	
Metals	Cd, Hg, Ag, Co, Pb, Cu, Cr, Fe
Organic	
Biodegradable	Sewage, domestic agricultural and process waste
Petrochemical	Oil, diesel, BTEX(Benzene, toluene, ethyl benzene, xylene)
Synthetic	Pesticides, organ halogens, polyaromatic hydrocarbon
Biological	
Pathogens	Bacteria, viruses
Gases	Sulphur dioxide, carbon dioxide, nitrous oxide, methane
Volatiles	Chlorofluorocarbon, volatile organic compound

Heavy metal pollution

The term heavy metal is used to describe metals with a density greater than 5 g/cm^3. Metals are introduced into aquatic system as a result of weathering of soil and rocks, from volcanic eruption and from variety of human activities. Continuous discharge of industrial, domestic and agricultural wastes in rivers and lakes causes deposit of pollutants in sediments. Such pollutants include heavy metals, which endanger public health after being incorporated in food chain. Heavy metals cannot be destroyed through biological degradation, as is the case with most organic pollutants. Incidence of heavy metal accumulation in fish, oysters, mussels, sediments and other components of aquatic ecosystems have been reported from all over the world (http://www.vigyanprasar.gov.in).

The primary source of heavy metal pollution in coastal lagoons are input from river sediments and atmosphere (http://as.iisc.ernet.in/energy/). River sediments, derived as a result of weathering are major carriers of heavy metals in the aquatic environment. Besides the natural processes, metal may enter into the aquatic system due to anthropogenic factors such as mining operations, disposal of industrial wastes and application of biocides for pests (Chakravarty and Patgiri, 2009).

Roadways and automobiles now are considered to be the largest sources of heavy metal pollution. Zinc, copper and lead are three of the most common heavy metals released from the road travels. Brakes release copper, while tire wear and tear releases zinc. Motor oil also tend to accumulate metals as it comes into contact with surrounding parts as the engine runs so oil leaks become another

pathway by which metals enter the environment. Most heavy metal are cations and carry positive charge. Soil particles and loose dust also carry charges. Most clay minerals have net negative charge. Soil organic matter tends to have a variety of charged sites on their surfaces, some positive and some negative. The negative charges of various soil particles tend to attract and bind the metal cations and prevent them from becoming soluble and dissolved in water. The soluble form of metal is thought to be more dangerous because it is easily transported and become readily available to plants and animals. In contrast, soil bound metals tend to stay in place. Metals can be transported by several processes. These processes are governed by the chemical nature of metals, soil and sediments particles and the pH of the surrounding environment. The aquatic environment is most susceptible to the harmful effect of the heavy metal pollution because aquatic organisms are in close and prolonged contact with soluble metals. pH is the measure of hydrogen ion concentration dissolved in water. H^+ is a cation which causes acidity. As a cation, it is attracted to the negative charges of the soil and sediment particles. In acid condition, there are enough H^+ ions to occupy many of the negatively charged surfaces of clay and organic matter. There remain a very less chance of keeping the metals bound and as a result, more metals remain in soluble phase. The effects of pH are even more pronounced in the Washington, DC metropolitan area because of the problem of acid rain. Acid rainfall causes a large increase in acidity and a corresponding increase in the amount of heavy metals becoming soluble (http//:www .fairfaxcounty.gov/nvswcd/newsletter/heavy metal.htm).

Indian scenario in heavy metal pollution

A preliminary survey of mercury in fishes from Bombay and Thane environment revealed the presence of these highly toxic metals in muscles, bones and brain in thirty species of fish from different sources. In Kalu river, sediments, soils and plants on the river bank showed fairly high contents of mercury, lead, cadmium and copper. Milk of buffaloes and cows of villages near the river were found with high concentration of mercury (http://www.vigyanprasar.gov.in). Several studies from water bodies near coastal Bombay, Baroda, Chandigarh, Lucknow, Chennai, Khetri complex, Raipur, Kanpur and river Cauveri nearby southern Karnataka have shown incidence of heavy metal accumulation in living matter. A study on Gandhisagar reservoir, Chambal river near Nagda and Kota, Khan river near Indore, Kshipra river near Ujjain and Lower Lake of Bhopal have shown accumulation of metal such as zinc, manganese, copper, nickel, mercury and lead in water, sediments and fishes (http://www.vigyanprasar.gov.in).

Metals toxicity

The ability of metals to disrupt the function of essential biological molecules, such as protein, enzyme and DNA is the major cause of their toxicity. Displacement of certain metal essential for cell by a similar metal is another cause of toxicity (Joshi, 2003).

Lead

The main sources of lead pollution are mining and smelting of lead ores, emission from automobile exhausts, use of glazed earthen ware containers, lead pipes and lead containers. The toxic effect of lead is

its interference with heme synthesis and leading to hematological damage. It also impairs the activity of porphobilinogen decarboxylase (Dara, 1993).

Nickel

Nickel is present in the effluent of silver refineries, electroplating, zinc base casting and storage of battery industry. The toxic symptoms are chest pain, nausea, vomiting, cyanosis, lung cancer and dermatitis (http://www.Biosorption -of –heavy- metals.htm).

Selenium

Selenium is an essential trace element, used particularly in the glutathione peroxidase enzyme system, which protect intracellular structure against oxidative damage. It can be dangerous in high amount. The symptoms of selenium toxicity are liver cirrhosis, neurological problem, gastrointestinal disturbance, pulmonaryodema, hair loss and sloughing of nails (http://www.medsafe.gov.nz/).

Chromium

Human are exposed to chromium through breathing, eating or drinking and through skin contact. Cr (VI) is dangerous to health and it causes alteration of genetic material and weakened immune system (http://www.Biosorption–of-heavy-metals.htm). Cr (VI) inhalation causes nosebleed, ulcer and holes in the nasal septum. Ingesting large amount of Cr (VI) can cause stomach upsets and ulcers, convulsions, kidney and liver damage and even death (Joshi, 2003).

Mercury

Mercury is generally considered to be one of the most toxic metal found in the environment. The major sources of mercury pollution in the environment are electrical and electronic, pharmaceutical, oil refineries, paper and pulp industries. The harmful effects of mercury are impairment of pulmonary function and liver, chest pain, chromosomal breaking and cell division. Methyl mercury causes deformities in offspring, mainly affect nervous system (http://www.Biosorption–of-heavy-metals.htm).

Arsenic

Arsenic compounds are highly toxic. The primary mechanism of arsenic toxicity is considered to result from its binding to sulfhydryl group of protein. Arsenate is known to affect oxidative phosphorylation by competition with phosphate (http://www.greenfacts.org/). Arsenite inhibits pyruvate dehydrogenases in the pyruvate acetyl CoA reaction (http://www.en.wikipedia.org/wiki/Arsenite). Acute arsenic poisoning from ingestion results in increased permeability of small blood vessels and inflammation and necrosis of the intestinal mucosa. These changes manifest as hemorrhagic gastroenteritis, hypotension, congestive heart failure, renal cortical necrosis, hyperkeratosis and pulmonary odema (http://www.wisegeek.com).

Cadmium

Cadmium is widely used in various alloy formulations, electroplating and in paints. Cadmium is highly toxic because of the absence of homeostatic control for this metal in the body. The symptoms of

cadmium toxicity includes irritation of respiratory and gastrointestinal tracts, liver injury, osteoporosis, damage to CNS, and immune system, psychological disturbance, cancer development, kidney damage and formation of kidney stones, glucosuria, proteinuria (Dara, 1993). Some of the industrial sites and contaminant are shown in Table 2.

Table 2. Industrial sites and contaminants (Scragg, 2005)

Industry	Sites	Contaminants
Chemical	Acid/ alkali works	Acid, alkalis, metals
	Dye works	Solvent, phenols
	Fertilizer and pesticides	Organic compounds
	Pharmaceutical	Organic compounds
	Paints and wood treatment	Chlorophenols
Petrochemical	Oil refineries	Hydrocarbons, phenols, acids, alkalis and asbestos
	Fuel storage	Hydrocarbons
	Tar distilleries	Phenols, acids
Metal	Iron and steel works	Metals especially Fe, Cu, Ni, Cr, Zn, Cd and Pb
	Foundries, smelters	
	Electroplating and galvanizing	
	Engineering	
	Shipbuilding	
	Scrap heaps	
Energy	Gas works	Phenols, cyanides, sulphur compounds
	Power station	Coal and coke dust
Mineral extraction	Mines and spoil heaps	Metals, Cu, Zn, and Pb
	Land restoration	Gas, leachate
	Quarries	Metals
Water supply and sewage	Waterworks	Metals and sludge
	Sewage treatment	Microorganisms, methane

Prevention of pollution with clean technology

Both national and international regulation will continue to put pressure on industries to minimize their impact on environment. Cleaner technology is about minimizing the environmental impact of release from processes. It is achieved by good engineering designs, good management practices and innovative process design. Integrated pollution control establishes a procedure for authorizing these activities and set a minimum requirement to be included in all permits. The aim is to prevent or reduce pollution of the atmosphere, water and soil as well as quantities of waste arising from industrial and agricultural installations, to ensure high level of environmental protection. In order to receive a permit for industrial or agricultural installation, they must comply with certain basic obligations like use all preventive measures, use energy efficiently, prevent all large scale pollution, waste management measures, emission limit value for polluting substances. "BATNEEC' (best available technology not entailing excessive cost) are used to prevent, minimize or render harmless releases of prescribed substances (http://www.rsc.org/ebooks/archive/free/BK9780854042104; http://europa.eu/legislation_summaries/)

Heavy metals enter waste waters from variety of sources, both domestic, industrial and from mining operations. Many of these dissolved metal ions such as copper, zinc, nickel etc are toxic to the living organisms. The most important feature that distinguishes heavy metals from other toxic pollutant is their non biodegradability. The toxicity due to metals ions is owing to their ability to bind with protein molecules and prevent replication of DNA and subsequent

cell division. Having entered into environment they play a significant role in aquatic ecosystem there by posing a biological threat to public health. Thus, there is concern for environment quality and to remove the heavy metals from waste water before its disposal. The removal of metals from waste water can be achieved by several physicochemical processes. The biological removal of heavy metal contaminates from aquatic effluents through bacteria offers great potential and therefore, microbiological approach to the problem cannot be neglected (Sahoo and Shukla, 1992). Heavy metals are known to have hazardous effects on human being, as depicted in Table 3.

Table 3. Toxic effects of heavy metals and their maximum contaminant level in effluents

Contami -nant	Maximum level (mg/L)	Sources	Effect
Arsenic	0.01	Natural deposits run off from orchards, glass and electronics production	Skin damage, increased risk of cancer, jaundice
Beryllium	0.004	Coal combustion, nuclear power plant, rocket fuel, electrical, aerospace and different industries	Acute and chronic respiratory diseases, lung cancer, beryllosis
Cadmium	0.005	Corrosion of galvanized pipes, natural deposits, metal refineries, phosphatic fertilizers, tobacco smoke, run-off from waste batteries and paints	Kidney damage, cardiovascular disease, hypertension, cancer
Copper	1.3	Pulp and paper, electrical goods, chemicals, corrosion of plumbing systems	Sporadic fever, pathological changes in brain tissue, gastrointestinal illness
Cyanide	0.2	Steel, metal, plastic, fertilizer factories	Nerve damage, thyroid problems
Fluoride	4	Water additive, natural deposits, fertilizer and aluminium factories	Bone disease
Chromium	0.1	Discharge from steel and pulp mills, natural deposits	Allergic dermatitis, ulceration and cancer
Mercury	0.002	Chlor-alkali industry, coal combustion, electrical batteries	Nervous failure, Renal disorder, minimata disease
Selenium	0.05	Petroleum refineries, sulphur and glass industries, instrument manufacturing, paper industry	Carcinogenic, hair/finger nail loss, circulatory problems
Lead	0.015	Battery industries, auto exhaust, paints etc.	Affect mental development in children and infants, behavioral disorder, cancer, constipation, blue line alang gums and death

Data adapted and modified from http://www.cheresources.com/biosorption.html; Scragg,2005

Possible solution to overcome metal pollution

Physical method

1. Coordination

A coordination complex is any combination of cations with molecules or pair of electrons. Bonding may be electrostatic, covalent or a combination is coordinately bonded to organic molecules (http://www.cheresources.com/biosorption.html).

2. Foam separation

Foam carrying metal is forced by a nitrogen gas to rise in a column and the foam is withdrawn in foam collector. This technique effectively recovers valuable metal solutes at low concentration in process streams. It is applicable to chromium, cobalt and nickel. (Shah, 2000).

3. Adsorption

In this process alumina, activated clay, bauxite, bone char, silica gel, synthetic polymers etc. have been used as principal adsorbent commercially in treating aqueous metal waste stream. Activated carbon adsorb hexavalent chromium, mercury and many metal compounds that have been complexed in the organic form as dye and pigments but granular carbon is preferred type. It is more expensive than powdered carbon but it can be chemically regenerated and reused. Powdered carbon is difficult to handle due to its tendency to dust and its removal is necessary from waste stream which is carried out by coagulation or by filtration (Shah, 2000).

4. Reverse osmosis

It is a process in which heavy metals are separated by a semi-permeable membrane by a pressure greater than osmotic pressure caused by dissolved solids in waste water. Cellulose acetate is promising material for the process but has disadvantage of being unsusceptible to various factors like temperature, pH and fouling. It is very expensive (Ahalya et.al, 2003 ; Shah, 2000).

5. Ultrafilteration

They are pressure driven membrane operations that use porous membrane for removal of heavy metals. Separation is based on solute size ranging from 2 to 10000 milimicron, depending on particular membrane porosity. Such membranes are very expensive. The main disadvantage is generation of sludge (Ahalya et.al, 2003).

6. Electrodialysis

In this process, ionic components (heavy metals) are separated through the use of semi-permeable ion or selective membrane. Application of electrical potential between the two electrodes causes migration of cations and anions towards respective electrodes. Because of the alternate spacing of the cations and anions permeable membrane, cells of concentration and dilute salts are formed. Electrolysis is used in metal plating industries; the disadvantage includes change in selectivity, voltage increase and problem of accumulation of suspended metal. Development is hindered by limitation on the life of membrane, the lack of economy of scale and high energy cost (Ahalya et.al, 2003; Patel, 2005).

7. Ion removal by rotating electrodes

In this process an ionized solution is passed into central chamber where it is contacted with two bipolar electrodes that adsorbs ions on the central chamber. This process has to be carried for series of times to achieve ion concentration upto required standard (Shah, 2000).

8. Cementation

This is a spontaneous process which involves the reduction of more electro-positive species by more electro-negative metal. Metal may be recovered from aqueous solutions by cementation with another metal having a high standard oxidation potential. In general metal will tend to precipitate any other metal with lower standard potential than the metal itself. Because of the formation of protective films and other barriers, every metal will not be recovered in this way (Shah, 2000; Patel, 2005).

Chemical methods

1. Ion exchange resins

It is a reversible chemical reaction, where an ion in a solution having similar charged ion, attach to an immobile solid particle. These solids are either naturally occurring inorganic zeolites or synthetically produced. Synthetic organic resins are the predominant type used today because they are tailored to specific applications. Ion exchange reactions are stoichiometric and reversible (http://www. cheresources.com/biosorption.shtml). It is an effective method for removing heavy metals from effluents. But the suspended solid in waste water can clog the exchanger and cause operational problem. This process is very impressive and gives high quality effluents. The

cost of this treatment is high (Shah, 2000). Ion exchange is a water treatment technology used to remove a variety of inorganic chemicals from contaminated groundwater. It removes unwanted ions such as chromium, iron, lead and nitrates in water and replaces them with less toxic ions such as sodium and chloride. Untreated water passes over an exchange material (an ion exchange resin) in tall column. As the water passes over the material, the unwanted ions attach to the resin releasing less toxic ions. Positive ions are often exchanged with sodium or hydrogen while negative ions are exchange with chloride or hydroxide. This method is effective in removing inorganic chemicals such as metal and non metal ions. The disadvantages are continuing costs are expensive, require continual monitoring of treated water to ensure that the exchange resin is not exhausted and require long term maintenance and regular inspections (www.hydroville.org/).

2. Chelation and extraction

The word "Chelation" is derived from the Greek word 'Chele', which means the firm binding of a metal ion with an organic molecule (ligand) to form a resulting ring structure that protects the mineral from entering into unwanted areas. Examples include carbonate and oxalate ions (http://www.cheresources.com/biosorption. shtml).

Chelation includes formation of metal chelates which are subsequently removed by solvent extraction. This method has drawback of input of lot of chemicals that may affect the economic viability of the method (Shah, 2000).

3. Chemical precipitation

The chemical used for precipitation of metals are lime, caustic soda, sodium carbonate and sulphides. Caustic soda is more expensive to buy as solid or concentrated liquor while lime is preferred because of low cost. Lime recover metals by forming their corresponding hydroxides besides lime, metal sulphides are excellent for removing metals but one of the disadvantage of using sulphide is that excess sulphides in solution will form hydrogen sulphide, which itself is a polluting compound and requires removal. Thus, precipitation of heavy metal from dilute solution is difficult, unless flocculating agent is used. But this can result in bulky wet sludge, which needs to be disposed off (Shah, 2000).

Drawbacks of conventional techniques of metal remediation

Conventional processes applied for remediation of metallic pollutants are often restricted due to technical and economical constraints (Dave, 2007). Some of them are:

1. Incomplete metal removal.
2. Need for expensive equipment and other related monitoring system.
3. High reagent or energy requirement.
4. It may result in secondary pollution due to generation of toxic sludge and other waste products that require separate disposal unit (Shah, 2000).

Microorganisms in abatement of heavy metal pollution

1. Bacteria

Bacteria resistant to heavy metals are frequently isolated from environmental sources such as soil and water. *Bacillus cereus, Mycobacterium scrofulaceum, Pseudomonas aeruginosa, Acidothiobacillus, Streptococcus agalactiae, Yersnia enterocolitica* and *Staphylococcus aureus* are reported to tolerate both cadmium and mercury. Bacterial species *Arthrobacter, Bacillus, Brevibacterium,* and *Cornybacterium* etc. absorb lead and mercury along with other heavy metals in the solution (Dubey and Maheshwari, 1999).

2. Actinomycetes

Actinomycetes *Flavoviridis* and several species of *Streptomyces* exhibited high ability to absorb mercury and lead along with the other heavy metals from mixed metal solution of manganese, cobalt, nickel, copper, zinc, lead and uranium also. *Streptomyces. Viridochromogenes* were shown to accumulate a large amount of uranium from aqueous systems (Dubey and Maheshwari, 1999).

3. Fungi

Heavy metal tolerance is a regular phenomena exhibited by a number of fungal species. Selective absorption of mercury and lead is reported from a mixed metal solution along with the other heavy metals by *A. niger, Fusarium oxysporum, Mucor hiemalis, Neurospora sitophila, Giberrella fujikuroi* and *Rhizopns oryzae* besides yeast species of the genera *Candida, Hensenula, Sacchromyces* and *Torulopsis*. *Macrophomina phaseolina* was reported to tolerate upto 500ppm of cadmium *in vitro* (Dubey and Maheshwari, 1999).

4. Cynobacteria

Several species of micro algae including green alga, *chlorella*, blue green algae, *Anabaena*, marine algae and macrophytes have been used for heavy metal removal. Biosorption of cadmium and nickel by capsulated cynobacteria has been studied (Dubey and Maheshwari, 1999).

Biological methods

1. Bioaccumulation

It involves the uptake of metals from contaminated media by living or dead organisms i.e. inactive biomass. Active plants and microorganisms accumulate metals as a result of normal metabolic processes via ion exchange, complexation reaction, intra and extra cellular precipitation at the cell walls (www.gwrtac.org).

2. Extracellular precipitation

Microbial activity is responsible for precipitation of metals in the form of hydroxides, carbonates, phosphates, sulphide and oxalates. Sulphate reducing bacteria *Desulfovibrio* and *Desulfotomaculum* are known to produce hydrogen sulphide as a byproduct of the metabolism, which reacts with soluble metal ions and convert them as insoluble metal sulphides. *Rhodotorula* sp. and *Trichoderma* sp. isolated from acid mine water are reported to precipitate copper due to hydrogen sulphide production (Dave, 2008).

3. Production of extracellular polymers

Many microorganisms secrete cell surface polymers in the form of capsule and slime layers, which are also called extracellular

polymers. Physical entrapment of precipitated metals in the polymer matrix and complexation of soluble species by charged constituents of polymers have been suggested to be important in metal removal. Depending on chemical composition they enable microorganisms to trap potentially toxic metals before their entry into plasma membrane. In *Saccharomyces cerevisiae*, it was found that under slightly acidic condition copper was chelated by peptides and proteins (Muraleedharan et. al, 1991; Shah 2000).

4. Metal transformation

Transformation of metals and metalloids are biogeochemically very important as they modify the mobility and toxicity of metalloids. It can be used to immobilize metals from surface and ground water. Microorganisms through their activity can change the oxidation state of several metals and turn them insoluble. Microbial catalyzed oxidation of iron by *Acidithiobacillus ferrooxidans* is the most important mechanism for removal of iron from acidic water treated by constructed wetlands. Microorganisms are responsible for oxidation-reduction, methylation and demethylation processes. Microbial oxidation of As^{3+} to As^{5+} and Fe^{2+} to Fe^{3+} helps in removal of arsenic and ferric iron by precipitation. *Leptothrix* and *Sphaerotilus* actively immobilize iron and manganese present in surface and ground water contaminated with biodegradable organics (Dave, 2008; Shah, 2000).

5. Metal precipitation

Microbiological metal precipitation is a common occurrence that is either the result of a dissimilatory reduction or the secondary

consequence of some metabolic processes, unrelated to the transformed metals (Bhattacharya and Banerjee, 2007).

6. The need for novel technology: Biosorption

Treatment of effluents with heavy metals following biotechnological approaches is simple, comparatively inexpensive and friendly to environment. Microbiological processes are of significance in determining metal mobility and have potential application in bioremediation of metal pollution (Jonglertjunya, 2008). Biosorption has been defined as the property of certain biomolecules (or types of biomass) to bind and concentrates selected ions or other molecules from aqueous solutions. Biosorption process is based mainly on the "affinity" between the biosorbent and biosorbate (Volesky, 2007). The term biosorption is defined as a process, in which solids of natural origin e.g. microorganisms, alive or dead, or their derivatives are employed for sequestration of heavy metals from an aqueous environment. Sources of biomass include (Murleedharan et. al, 1991).

1. Sea weeds
2. Microorganisms (Bacteria, fungi, yeast and molds)
3. Activated sludge
4. Fermentation waste
5. Other especially propagated biomasses (http://www.cheresources.com).

Metal removal processes based on biosorbent properties of microbial biomass that can be used for metal removal from waste waters (Puranik and Paknikar, 1999). Origin of biomass is a major factor in biosorption process e.g. bacteria, yeast, fungi and algae coming from

their natural habitats are good sources of biomass and fast growing organisms that are specifically cultivated for biosorption purpose. Apart from the microbial sources even agricultural products such as wool, rice, straw, peat moss, exhausted coffee, waste tea, walnut skin, coconut husks, and husk of Bengal gram were also used. Metal biosorption is dependent on the status of biomass (living and non-living) types of materials used, properties of metal solution chemistry, ambient / environmental condition such as pH, temperature, salinity etc. influence the mechanism of metal biosorption (Das et. al, 2008). Diversity in the cell wall composition among the different biomass like algae, fungi, yeast and bacteria cause significant differences in the type and amount of metal ion binding to them. Fungal cell wall contain chitin, chitosin and bacterial cell wall composed of peptidoglycans, which consists of linear chain of disaccharide N- acetylglucosamine, β-1, 4-N-acetylmuramic acid with peptide chains (Das et.al, 2008) and algal cell wall made up of cellulose, which provide carboxylates, amines, phosphates, sulfates, hydroxyls for metal binding (Damani, 2009).

There is a wide variety of microorganisms that can interact with metals and radionuclides and transform them such as *Citrobacter* sp. for lead and cadmium, *Bacillus subtilis* for chromium and *Pseudomonas aeruginosa* for uranium (http://www.cheresources.com/ biosorption). Bacterial biosorbents have now entered the market for removing metals from industrial effluents and remediation of contaminated ground water. The inherent ability of bacterial cells to accumulate, bind, precipitate and transform metals has been coupled with cell immobilization and engineering system to yield a robust

and versatile technology for metal treatment (Ehrlich and Brierley, 1990).

Biosorption is an ideal process for the treatment of high volume low concentration complex waste (Dave, 2008). The internal and external sequestration of metals means that biological material can bind metals to high level of up to 30% of dry weight. It has been shown that biosorption may be economically competitive with chemical techniques, particularly when the biomass used is inexpensive such as waste biomass from the fermentation industry, excess sewage sludge and easily harvested mine and easily harvested marine algae (Scragg, 2005).

For attaining good biosorption property the following criteria have been selected for a better biosorbent (Muraleedharan et.al, 1991):

1. The active biosorbent should be produced at low cost and should be usable.
2. Particle size, shape and mechanical properties should be suitable for use in a continuous-flow system in completely mixed, packed or fluidized bed reactor configurations.
3. Uptake and release of the metal should be efficient and rapid.
4. Separation of the biosorbent from solution should be economical, efficient and rapid.
5. It is desirable that the sorbent is metal selective.
6. Desorption of metals should preferably be metal selective and economically feasible and sorbent should remain in physical state that can be reused.

The uptake of metals from wastewater by living material can be active or passive. Passive uptake is independent of cellular metabolism involves the binding of metals to the polyanionic cell wall or by ion exchange with ions in the cell wall. Passive uptake is fast, reaching completion in a very short period and unaffected by metabolic inhibitors, but is affected by physical condition such as pH and ionic strength. Passive binding is reversible and can occur with both living and dead material. Passive biosorption proceeds rapidly by any metal binding mechanism such as coordination, complexation, ion exchange, physical adsorption or inorganic microprecipitation. Passive mode of sorption is independent of energy, mainly through chemical, functional groups of the material, comprising the cell and particularly cell wall. Active uptake is slower than passive uptake, dependent upon cellular metabolism, and is affected by metabolic inhibitors, uncouplers and temperature. In active uptake the metals are complexed with specific proteins, such as metallothioneins, or contained in the vacuole (Scragg, 2005).

Advantages of biosorption

1. Biosorption is highly competitive with current technologies such as ion exchange, electro dialysis, reverse osmosis etc. It gives competitive performance. However, it has early saturation point i.e. when metal interactive sites are occupied, metal desorption is necessary for further uses (Damani, 2009).
2. Renewable biomaterials, which reduce production cost, fast adsorption kinetics (Mohapatra, 2002).
3. High selectivity of biosorbents-possible to recover valuable metals, separation of mixtures (Damani, 2009).

4. Low operating cost and can be operated at ambient conditions of pH and supply of nutrients is not required (Joshi, 2003).
5. Biosorption has a distinct advantage over conventional methods such as no chemical sludge generation takes place, metal recovery is possible and process equipments are known. It is cost effective for treatment of large volume of waste water containing low metal concentration (Puranik and Paknikar, 1999).
6. The higher specificity of biosorbents never allows them to be overloaded with alkaline earth metals, a very common problem with chemical techniques such as ion exchange resins. Genetic modifications can result in strain improvement, which would enable increased bioaccumulation, production of new metal chelating peptides (Bhattacharya and Banerjee, 2007).

Disadvantages of biosorption

1. Early saturation i.e. when metal interactive sites are occupied metal desorption is necessary prior for further use.
2. The potential for most biological process improvement is limited because cells are not metabolizing (Damani, 2009).

Commercial application of biosorption

Some of the metal sequestering biosorbent have been commercialized by doing critical analysis of different microbial masses. Some excellent products based on immobilized biomass such as, AMT-BIOCLAIM™, AlgaSorb™, Bio-Fix and BIOMAT® have been developed, patented and commercialized for detoxification of metal ions from industrial waste or effluents (Dave, 2008). This is important in present scenario, as their effective, economical and viable process

to remove metal ions from industrial waste water and drinking water. AMT-BIOCLAIM™ process was used in the form of fixed bed reactors containing 20 kg of granular biosorbents and was reported for removing Cd, Cr, Cu, Hg, Ni, Pb, and U. AlgaSorb, a potent biosorbent successfully used to remove Ag, Al, Au, Cu, Co, Cr, Hg, Ni, Pb, Pt, U and Zn from contaminated effluents and process streams using column reactors. This can efficiently remove metallic ions from dilute solution i.e. 1-100 mg/L and reduces the concentration of metals down to 1 mg/L or even below (Dave, 2008; Damani, 2009; Mohapatra, 2002). Following are some patents for biosorption are depicted in Table 4. Microorganisms used in patented products for metal remediation are shown in Table 5.

Table 4. List of US patents on biosorption (Mohapatra, 2002)

Patent No.	Title of the patent	Year	Author
4,898,827	Metal recovery	1990	Brierley et al.
5,0554,2	Removal of metal ions with immobilized metal ion-binding microorganisms	1991	Greene et al.
5,538,645	Process for the removal of species containing metallic ions from effluents	1996	Yannai et al.
5,648,313	Method for production of adsorption material	1997	Pohl

Table 5. Microorganisms used in patented products for metal remediation (Dave, 2008)

Patent product	Micro-organism used
AMT-BIOCLAIM™	*Bacillus*
AlgaSorb™	Fresh water algae
Bio-Fix	Yeast, alga, plants and bacteria
BIOMAT®	Cyanobacteria

7. Phytoremediation

Phytoremediation is an emerging technology based on the use of plants to clean up polluted sites. It refers to the specific ability of the plants to aid in metal remediation. Most metal uptake occurs in the root system, usually via absorption, where many mechanisms are available to prevent metal toxicity due to high concentration of metals in the soil and the water. *Thlaspi caerulescens* accumulate zinc and cadmium. *Alyssum lesbiacumaccum* accumulate nickel (Bhattacharya and Banerjee, 2007; http://www.gwrtac.org).

8. Phytoextraction

It is uptake of metals and organic pollutants by the roots and shoots of the plants and their storage in roots, leaves and stems e.g. sunflower roots can concentrate uranium 30,000 fold from contaminated water (Scragg, 2005).

9. Phytostabilization

Phytostabilization makes use of immobilization and reduction in the mobility and bioavailability of contaminants by plant roots and

associated microbes. Some grasses have been made commercially available for phytostabilization of metals like lead, copper, zinc. Nickel has been removed from plating wastes by bacteria and other organisms are being genetically engineered to remove metals such as cadmium, cobalt, copper and mercury (Bhattacharya and Banerjee, 2007; http://www.gwrtac.org).

Search for metabolic organism for bioremediation of metallic pollutants

Iron precipitating organisms

Importance of iron

Iron is important biologically. Cells use it catalytically in the enzymatic transfer of electrons in respiration and photosynthesis. Cells also employ iron in the heme group of enzymes catalase and peroxidase, which catalyze reaction involving hydrogen peroxide. (Ehrlich, 1981). Iron is commonly found in rocks and soil. Under proper conditions, iron will leach in the water resources from rock and soil formation. Exceeding iron concentration greater than 0.3 mg/L causes water staining that adversely affect plumbing fixtures, dishware and clothes that produce a yellow to reddish appearance in water (Shokoohi et.al, 2009).

Microbial activities in iron geochemistry

The type of demand for nutritional requirements determines the microbial activities in the iron geochemistry are as follows:

1. Digestion of organic-metallic complexes

The organic part of organic-metallic complexes, such as carbon, nitrogen may be used by some bacteria or fungi, releasing inorganic part to the medium, thus making them free to undergo chemical transformation. The heterotrophic bacteria sticks on surfaces and assimilates the organic part of the organo-iron complexes, releasing other ferrous and ferric ions for precipitation.

2. Products of metabolism

Product released by the microorganisms can create reducing or oxidizing microenvironments which contribute to solubilization or precipitation of certain elements.

3. Surface cell absorption

Microorganisms keep certain elements adsorbed in their cell wall, facilitating microbial or chemical action on them.

4. **Acquisition of energy (Chemotrophic)**

Specific enzymes synthesized by the microorganisms, act as a catalysts and enormously increase the reaction speed to meet their energy demands (Mendonca et. al, 2003)

Microorganisms play an important role in the natural environment by determining the speciation of iron, they can also cause considerable iron accumulation through biomineralization. The most common electron acceptors in natural environment is iron and because of its widespread abundance groundwater are generally reduced due to the activity of the iron reducing bacteria. Iron

precipitation was regarded by most geologists as a chemical process. As per the literature, Ehrenberg (1836) was the first to put forward the suggestion that biological processes are important in the deposition of iron rich sediments. As per the literature, Schwertmann and Taylor in 1989 and Lovely in 1991 have shown that actual contact with the bacterial cell is required for enzymatic reduction to occur. As reported by Staurt and Olli, Starkey (1945) studied the ecology of iron transformation in water and stated that interaction among filamentous iron bacteria, sulfur oxidizing bacteria, sulfate reducing bacteria and heterotrophs involved directly or indirectly in the precipitation of iron (Staurt and Olli, 1985).

Iron electrochemistry

Element is reduced or precipitated depends on electrochemical potential (Eh), partial oxygen pressure, pH, temperature and pressure. According to Figure 3, under very low electrochemical potential the iron is found as an immune metal (Fe). When (Eh) increases, iron oxidizes, changing to ionic form of Fe^{2+}, and stays soluble. If (Eh) increases Fe^{2+} is further oxidized to Fe^{3+} and precipitate as follows in which Fe^{2+} is dissolved and Fe_2O_3 is in precipitate state.

$$2Fe^{2+} + 3H_2O \rightarrow Fe_2O_3 + 6H^+ + 2e^-$$

Iron may be associated with organic matter, forming organic metallic complex. Under these condition the ion Fe^{2+} or Fe^{3+} is not influenced by environmental condition and may stay dissolved in water, even at conditions under which dissolved ionic iron precipitate. Only under

extreme acidic condition is it possible to dissociate the link between the iron and organic matter to destroy the complex.

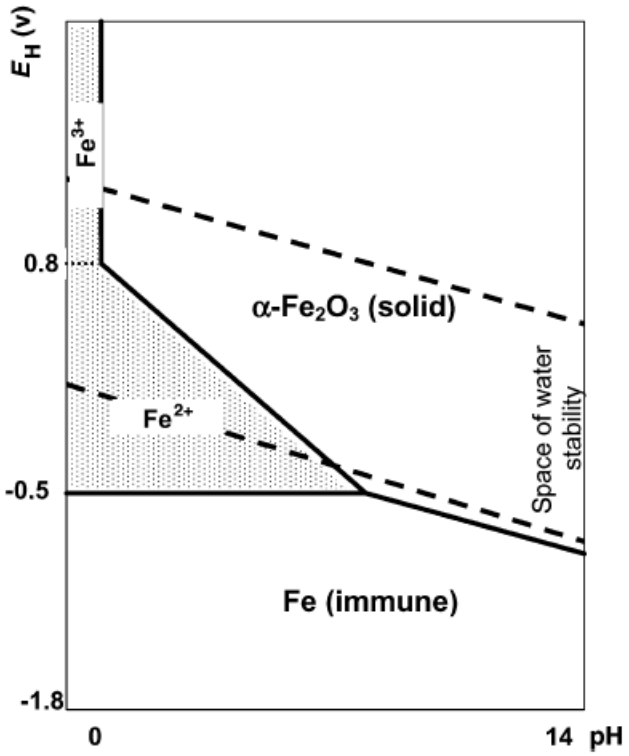


Figure 3. Typical diagram of iron electrochemistry equilibrium (Mendonca et.al, 2003**)**

Chemical mechanism of iron precipitation

Iron is found in number of minerals, in rocks, soils and sediments. It can exists in oxidation state of 0, +2, and+3. At pH values greater than 5, its ferrous form is readily oxidized in air to the ferric form. Under reducing conditions, ferric iron is readily reduced to ferrous state. In acid solution, metallic iron readily oxidizes to ferrous iron with the production of hydrogen. Ferric iron precipitate in alkaline solution and dissolves in acid solution (Ehrlich, 1981).

Some microorganisms can catalyze iron precipitation that enormously increase in the reaction speed of the process (Mendonca et.al, 2003). The iron precipitating bacterium *Gallionella ferruginea* was found to dominate the biotic iron oxidation/precipitation process in a sand filter of fresh water treatment plant. The precipitate Fe oxide was found to be Ferrihydrate (Sogaard et. al, 2001).The most effective iron precipitating bacteria in drain pipes have been from group consisting of long filaments such as *Gallionella* and *Sphaerotilus*. There are certain rod bacteria, such as *Pseudomonas* and *Enterobacter* that can also precipitate iron. (http://www.edis.ifas.ufl.edu). In some instances the deposition of iron may arise from enzymatic mechanism that brings about the oxidation of ferrous iron in solution. However, it may also result from non enzymatic reactions followed by adsorption of the iron to the surface of microbial cell (Macrae and Edwards, 1972). The main pathways of microbial accumulation of Fe oxides are as follows:

1. Oxidation of ferrous oxides by metabolic products.
2. Deposition of iron as a result of utilization of the organic portion of its complex or chelate compounds.
3. Chemosorptional phenomena on the cell surface (Dubinina and Balashova, 1985).

Iron bacteria have the ability to assimilate dissolved iron which they oxidize or reduce to ferrous or ferric ions for energy. The ions are precipitated as hydrate ferric hydroxide or in their mucilaginous sheath (http://www.140,194,76,129/publications). “Iron Bacteria” is the collective name for a large and a diverse group of bacteria, which

have an important impact on water treatment and distribution systems (Eaton et al., 1995). Iron bacteria derive their energy for growth from the oxidation of the soluble ferrous iron (Fe^{2+}), present in ground water, to its insoluble ferric form (Fe^{3+}). The result is an accumulation of iron precipitates, which along with the bacteria are formed into biofilms (http://www.samdbnrm.sa.gov.au/Portals/9).

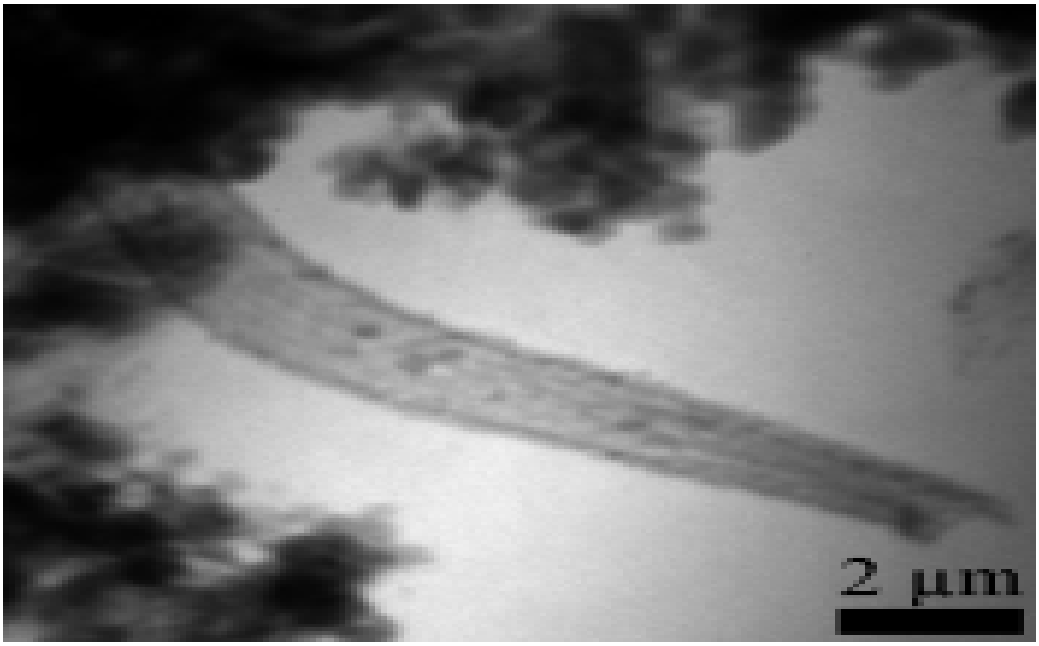


Figure 4. Image of iron precipitating bacteria
http://www.isa.au.dk/facilities/astrid/beamlines/xrm/xrm3.asp

Bacteria are also responsible for the precipitation of iron deposits in Danish Wetlands in Danish called Myremalm. These studies are in collaboration with the Department of Earth Sciences, University of Aarhus and with the Department of Chemistry (http://www .isa.au.dk/). Image of iron precipitating bacteria is shown in Figure 4.

Classification of iron bacteria based on Bergey's manual of systematic bacteriology

In *Bergey's Manual of Systematic Bacteriology* (Volume 3), the iron bacteria were listed in Section 21 : Budding or Appendaged bacteria and included *Pedomicrobium, Gallionella, Metallogenium* and *Caulobacterx*, in Section 22 : Sheathed Bacteria which included *Sphaerotilus, Leptothrix, Lieskeella, Crenothrix* and *Clonothrix,* in Section 23 : Non photosynthetic, Non fruiting Gliding Bacteria such as *Beggiatoa, Thiothrix, Toxothrix, Thiobacillus* (one species only, *Thiobacillus ferrooxidans* and in Section 20 : Aerobic chemolithtrophic Bacteria and Associated organisms including *Siderocapsa, Naumanniella, Ochrobium* and *Siderococcus* (Staley and Furest, 1989; Larkin, 1989; Mulder, 1989)

A system of classification based on the physical form of iron bacteria has been employed by water well industry. The three general forms recognized are:

1. ***Siderocapsa***

   The organism consists of numerous short rods surrounded by a mucoid capsule. They deposit hydrous ferric oxide rust brown precipitate.

2. ***Gallionella***

   This organism is composed of twisted stalks or bands which is the only living part of the organism, and found at the end of the stalk.

3. ***Filamentous Group***

   Filamentous group consist of four genera;

a. *Sphaerotilus*
b. *Crenothrix*
c. *Clonothrix*
d. *Leptothrix*

The organisms are structurally characterized by filaments which are composed of series of cells enclosed in sheath. The sheath is commonly covered with slime layer. These organisms typically become encrusted with ferric hydrate resulting in large masses of filamentous growth and iron deposits. (www.140,194,76,129 /publications/).

Iron reduction

The form of iron depends upon the ratio of iron/carbon as citrate. If I:C > 1:6, ferrous minerals, such as siderite, ferric hydroxide are formed. If I:C < 1:6, then ferric minerals mainly ferrihydrite altering to hematite are precipitated. This suggests that two different method of iron reduction may be operating with greater concentration of citrate. NMR spectra show that iron is reduced whilst still chelated, but with the lesser concentration the citrate is rapidly consumed and the iron is precipitated as Fe^{3+} before being reduced to Fe^{2+}. Citrate is necessary for reduction of iron (Sherriff and Brown, 1999). This iron reduction is observed in nature, where a consortium of microorganism is present sometimes in biofilm formation.

Biofilm

The organisms comprising a biofilm that occurred in URL (Underground Research Laboratory) excavated by Atomic Energy of Canada. By phospholipids analysis of consortium it was found that

that majority of the bacteria were Gram negative with only few Gram positive bacteria. The known dissimilatory iron reducing bacteria *Shewenella* sp., sulphate reducers, *Desulfovibrio* sp., *Pseudomonas* and *Actinomycetes* were also present. One of the main reactions of consortium is the ability to reduce iron. This reaction appears to be widespread. In URL bacteria and as well as from an iron precipitation steam in English Weald, after 24 transfers without iron, were still able to rapidly reduce iron. Iron is only soluble at low pH, so that at natural pH of 8.5 to 9 found in the Shield groundwater it must be chelated to be in solution. Citrate is used to chelate iron and metabolized as carbon source by the bacteria, which destroy the chelation and allow the iron to be precipitated (Sherriff and Brown, 1999).

Non enzymatic iron oxidation

As reported by Ehrlich, Harder (1919), Winogradsky (1922), Starkey and Halvorson (1927) have concluded that any organism, which raises the pH of a medium by forming ammonia from protein or protein derived material or by consuming salts of organic acid can promote ferrous iron oxidation. Ferrous iron may be protected from chemical oxidation at elevated pH and Eh by chelation with oxalate, citrate and humic acids. Bacterial breakdown of the ligand will free the ferrous iron, which then oxidizes spontaneously to ferric iron. This has been demonstrated in the laboratory with *Pseudomonas* and *Bacillus* strain. These cultures do not derive any energy from iron oxidation but rather from the oxidation of the ligand (Ehrlich, 1981).

Other genera reported for iron precipitation

As reported by Cullimore and McCann, Clark et al. (1967) briefly reviewed the definition of the term iron bacteria and considered that it included all organisms capable of precipitating iron biologically. Using synthetic media containing ferric ammonium citrate, they found that the isolates of *Aerobacter aerogens, Serratia indica* and *Bacillus pumulis* could all precipitate iron mainly through the utilization of citrate (Cullimore and McCann, 1978). They also reported that heterotrophs such as *Aerobacter (Enterobacter) aerogenes* were usually found in association with the classical iron bacteria and participated in the iron precipitation. An iron bacterium is a concept encompassing many genera and species of bacteria with varying morphology and physiology. Iron precipitation and accumulation are not limited to these iron bacteria. Ferric iron precipitation may result from microbial destruction (mineralization) of the chelators. A variety of microorganisms including bacteria *Gallionella* sp., *Leptothrix, Pedomicrobium, Naumanniella*, *Siderocapsa* and protozoans such as *Anthophysa, Siderodendron, Bikosoeca,* and *Siphonomonas* have been found capable of removing ferric iron from solution by adsorption to surfaces of cells or to inanimate matter. Precipitation of ferric iron is due to the biological destruction of the ligand of a ferric iron complex. The intact ligand keeps ferric iron in solution. Ferric iron reduction was reported in bacteria such as *B. polymyxa* and *B. circulans*, *Escherichia freundii* and *Paracolobactrum*. Hematite reduction by fungi *Alternaria tenuis, Fusarium oxysporum* and *Fusarium solani* were also reported. Not all microbial ferric iron reduction is enzymatic. Some may be the result of reaction with metabolic end

products such as hydrogen sulphide or formate. Both oxidative and reductive reactions of iron by microbe play important role in the iron cycle in nature (Ehrlich, 1981).

Growth characteristics

Pseudomonas, Moraxella, Alcaligenes and *Acinetobacter* were reported for their rapid deposition on ferric ammonium citrate agar. All of these strains produced dark brown colonies on ferric ammonium citrate agar, indicating the utilization of citrate. At the time of inoculation with isolates the medium was a clear yellow liquid with a pH of 7.0. During incubation, changes in the appearance of the culture were observed. After 12 d of inoculation, *Morexlla* strain 2 and *Pseudomonas* strain 3 produced a heavy, orange red precipitate in the medium, leaving a water clear supernatant fluid. The pH of the media after incubation was 8.2. The colour and pH of uninnoculated sample remain unchanged during the incubation period (Macrae et.al, 1973). Growth of isolates in liquid ferric ammonium citrate are shown in Table 6.

Table 6. Growth of isolates in liquid ferric ammonium citrate medium

Organism	Incubation time (d)			
	0	6	12	34
Morexlla strain 2	Clear yellow	Orange red	Orange red precipitate	Orange red precipitate
Pseudomonas strain 3	Clear yellow	Orange red	Orange red precipitate	Orange red precipitate

Dissimilatory iron reducing bacteria

Laboratory studies have demonstrated the potential for Fe^{3+} reducing microorganisms to remove uranium from contaminated ground waters Fe^{3+} reducing microorganisms can immobilize a variety of contaminant metals and metalloids by reducing them to less soluble forms (Lovely and Anderson, 2000). Microorganisms that use metals as terminal electron acceptors, or reduce metals as a detoxification mechanism, have an important influence on the geochemistry of aquatic sediments, submerged soils and the terrestrial subsurface. Furthermore, it is becoming increasingly apparent that microbial metal reduction may be manipulated to aid in the remediation of environments and waste streams contaminated with metals and certain organics. Microbial reduction of ferric iron to ferrous iron has been studied not only because of its influence on iron geochemistry but also because ferric iron is one of the most abundant potential electron acceptors for organic metal decomposition in many aquatic sediments and subsurface environment. Some of the ferric reducing microorganisms are *Geobacter metallireducens, Shewanella putrefactions* etc. Dissimilatory Fe^{3+} reduction has a greater overall environmental impact than microbial reduction of any other metal. Microbial Fe^{3+} reduction is important in following phenomenon: organic matter decomposition in variety of fresh water and marine sediments, the oxidation of organic coupled to Fe^{3+} that resulted in the accumulation of magnetite in the banded iron formation, the formation of other ferrous iron minerals such as siderite and vivianite (Lovely, 1993)

It was proposed that iron deposition may represent a kind of iron detoxification mechanism for *Sphaerotilus*. Although far from removing significant quantities of this element, iron deposition could provide protection either by changing the microenvironment around the cell and/or its surrounding sheath less permeable to iron, or by preventing access of toxic metal to the cell membrane. These processes could increase the organism's tolerance to high concentration of the element (Rogers and Anderson, 1976). Ferrous iron may serve as major energy source to certain bacteria and ferric iron may serve as terminal electron acceptor for some other bacteria. Large scale microbial iron oxidation and reduction are important because it leads to precipitation and solubilisation of iron in the biosphere. Adsorption of iron to bacterial surface is known to occur and is possible precursor to biomineralization of iron oxides. The stability of the iron bacteria sorption reaction is orders of magnitude stronger than that observed for the other metal bacteria systems, emphasizing the importance of Fe^{3+} adsorption in bacteria bearing systems (Wightman and Fein, 2004).

Application of iron precipitating microorganisms in metal removal

Dissimilatory iron reducing bacteria such as genus *Geobacter* and *Pyrobaculum islandicum* reduced toxic metals Co^{3+}, U^{6+}, Tc^{7+} and Cr^{6+}. *Sulfurospirillum barnesii* reduced and mobilized oxidized As^{5+} that was coprecipitated on ferrihydrite. Citrate has found use as a chelating agent in decontamination operation, forming highly recalcitrant and mobile metal citrate complexes. *Pseudomonas aeruginosa, Pseudomonas putida* were able to grow in metal citrate

complexes utilize as carbon sources, metal precipitation promoted by the addition of inorganic phosphate. A unique aspect of this study was the use of *Pseudomonas putida* to treat Ni citrate waste to remove nickel (Lloyd and Lovely, 2001). Living cells of *Sphaerotilus natan* are used for heavy metals cadmium, copper, zinc, chromium removal from aqueous solution (Lodi et.al, 1997).

Chapter 2

Aims and Objectives

In this context, the research work has been undertaken with the following objectives:

1. Isolation of iron precipitating bacteria.
2. Screening of different isolates on the basis of iron precipitating capacity.
3. Study of resistance in selected isolates for copper, cadmium, cobalt and arsenic.
4. Identification of the selected bacteria.
5. Optimization of iron bioprecipitation study.
6. Laboratory scale study for the bioremoval of copper, mercury and cadmium from aqueous solution.
7. Selenium reduction and manganese oxidation study.

Chapter 3

Iron Bioprecipitation

Introduction

Iron is the fourth most abundant element in the first transition series, and the most common element in the whole planet. Iron exists in wide range of oxidation state -2 to +6, but +2 and +3 are the most common. (www.en.wikipedia.org/wiki/Iron). Iron is silvery white or grayish metal. It readily combines with oxygen in moist air to form iron oxide (Fe_2O_3) known as rust (www.chemistryexplained.com).

Occurrence

The large iron sources in the world are in China, Russia, Brazil, Canada, Australia and India. The most common ores of iron are hematite, limonite, magnetite, siderite and iron carbonate (www.chemistryexplained.com). Properties of iron and image of its mineral are shown in Table 7 and Figure 5.

Figure 5. Image of iron mineral (http://www.amazingrust.com /experiments /how_to/Images/Iron_ Lump.jpg

Table 7. Properties of iron

Property	Specification
Atomic number	26
Atomic mass (g/mol)	55.85
Electro negativity according to Pauling	1.8
Density (g/cm^3 at 20 ºC)	7.8
Melting point (ºC)	1536
Boiling point (ºC)	2861
Vanderwaals radius (nm)	0.126
Ionic radius (nm)	0.076 (+2); 0.064 (+3)
Isotopes	8
Energy of first ionization kJ/mol	761
Energy of second ionization kJ/mol	1556.5
Energy of third ionization kJ/mol	29511
Standard potential (Fe^{2+}/Fe)	-0.44 V
(Fe^{3+}/Fe^{2+})	0.77 V
Discovered by	The ancients

Data adapted and modified from (http://www.lenntech.com/periodic)

Functions

Iron is essential to almost all living things from microorganisms to human beings. Iron is an important constituent of the haemoglobin molecule that transports oxygen from lungs to all body tissues (www.lenntech.com/periodic/elements/fe.htm). Iron is necessary for brain development, regulation of body temperature, muscle activity and catecholamine metabolism (http://www.health vitaminsguide.com).

Applications

Iron is one of the most useful metal. Iron sulfate is used as fungicides and oxalate of iron is used in photographic development. Magnetite is used in the production of industrial electrodes. Iron carbonyl is used as catalyser in many reactions (http://nautilus.fis.uc.pt/st2.5).

It is also used in the frames of heavy carriers like ships, heavy vehicle and machinery, framework of many buildings including skyscrapers and bridges. Iron is also used as chemical in the making of various types of dyes, paints and pigments (www.buzzle.com/articles).

Iron Toxicity

Chronic inhalation of excessive concentration of iron oxide fumes or dusts may result in the development of benign pneumoconiosis, called siderosis (www.lenntech.com/periodic/elements/fe.htm) Haemochromatosis, the most common form of iron overload disease, is an inherited disorder that causes the body to absorb and store too much iron. The extra iron build-up in the organs may eventually lead to serious problems such as liver cirrhosis, heart abnormalities, thyroid deficiency and damage to adrenal gland and pancreas (www.medic8.com/healthguide/articles).

Iron bioprecipitation

Bacteria possessing the iron precipitating characteristics had two features in common viz. the ability to utilize citrate and possession of capsular material (Cullimore and McCann, 1978).

The most important metabolic processes for immobilization of metal/metalloid and radionuclide species are bioprecipitation. Bioprecipitation include the transformation of soluble species to insoluble hydroxides, carbonates, phosphates or sulphides as a result of microbial metabolism. In case of biological reduction, the cells use the species as terminal electron acceptors in anoxic environment to produce energy and to reduce the toxicity of the cell's microenvironment (Tsezos, 2009). Iron bacteria obtain energy by the oxidation of iron from the ferrous to ferric state which precipitates as ferric hydroxide around cells in citrate agar. (http://www.himedialabs.com/TD/ M728.pdf). Some bacteria that do not oxidize ferrous iron may dissolve or deposit it indirectly. During their growth, they either liberate iron by utilizing organic radicals, to which iron is attached or alter environmental condition to permit deposition of iron (http://www.himedialabs.com /TD/M622.pdf). These bacteria are usually non filamentous, spherical or rod shaped. Bacteria frequently thrive in iron bearing water and form reddish brown growth that may clog pipes and reduce flow rates (www.freedrinkingwater.com).

The most common habitat of iron bacteria includes slowly flowing water such as streams and drainage ditches or stagnant water of pools or ponds. In iron precipitating organisms iron deposition system might include a portion of the protein polysaccharide lipid complex, comprising the sheath material of the organisms. Once this material is synthesized by the bacterium it may act in autonomous manner facilitating iron deposition (Roger and Anderson, 1976).

Figure 6. Image of ferric precipitate (http://www.umaine.edu /WaterResearch/FieldGuide/webphotos/orangeslime.jpg)

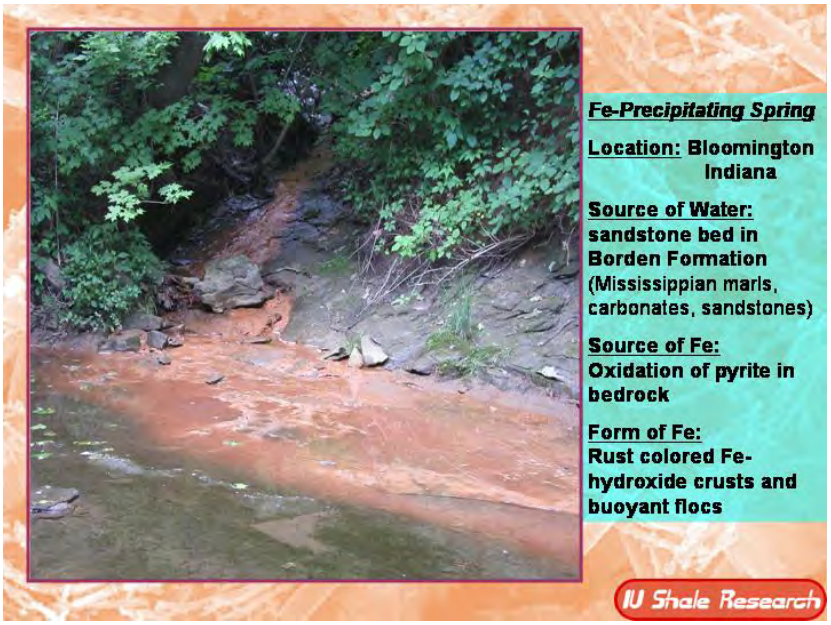


Figure 7. Image of Fe-precipitating spring. (http://www.shale-mudstone-research-schieber.indiana. edu/images)

Sphaerotilus, Leptothrix, Crenothrix, Clonothrix and *Gallionella* are common type of iron bacteria. These bacteria can live in wide range of environment condition (www.wcponline.com). The role of bacteria in iron precipitation was indirect as they change iron speciation and

saturation conditions. Some heterotrophic bacteria may precipitate ferric iron via utilization of carbon from organic complexes of Fe^{3+}. For monitoring of iron precipitating microorganisms, a differential solid medium was developed from the general purpose heterotrophic plate count medium by incorporating ferric ammonium citrate in the formulation. Organisms capable of using citrate in the modified medium yield rust coloured colonies due to the formation of Fe^{3+} precipitate (Tuhela et.al, 1993).

Biological methods of iron removal with bacterial strains like *Pseudomonas* sp., *Moraxella* sp. and *Agrobacterium* sp. use synthetic media incubated at 25-30 °C and pH 5.5-7.2. *P. fluorescence* is reported to be capable of complete utilization of ferric citrate in media containing 280 mg/L Fe^{3+}. Iron bioprecipitating organism utilizes the organic moiety of the complexes as carbon sources, there by releasing the iron which precipitate as ferric hydroxide (Gopalan et.al, 1993).

Aspects of iron precipitation in microbial mats

The Borra caves, Vishakapattanam in India has significant amount of unexplored microbial mats in spring waters. The spring water having pH 7.5 - 7.7 contains dissolve metals like iron and organic mat sludge. On the basis of direct microscopy and SEM, species of *Leptothrix, Gallionella* and some additional bacteria were observed at Borra caves habitat. This study indicates that the presence of these iron rich mats in the spring water could be linked to the presence of abundant active communities of iron precipitating bacteria at Borra caves (Baskar et.al, 2007).

The oxidized form of iron is amorphous ferric hydroxide, which is known to remove zinc and other heavy metals from water (Leake, 2009). Adsorption behaviour of amorphous hydroxide type adsorbents, i.e. ferric hydroxide, ferric cupric hydroxide and ferric lead hydroxide, were reported for removing As^{3+}, Se^{6+}, Mo^{6+} and Sb^{3+} ions from aqueous solution (Fujita , 2006). In this context, aims were isolation, screening and identification of iron bioprecipitation process.

Materials and methods

Sample collection

Water and soil sample were collected from four different places viz. Vadodara, Gandhinagar and Ahmedabad districts of Gujarat and Raichur district, Karanataka. The sampling sites are listed in Table 8. The samples were transported in possible minimum time (12 to 48 h) and were preserved at 5±1 ºC until further processing.

Physico-chemical parameters

Physico chemical parameters of all the samples were studied in terms of pH, oxidation-reduction potential, conductivity, salinity and total dissolved solids (TDS). The pH was measured using digital pH meter (Systronics 361, India). Redox potential, conductivity, salinity and TDS were measured using portable meters (Eutech, Singapore).

Isolation

Isolation of iron precipitating cultures was done from different samples by streaking citrate agar plates (Appendix I) and well isolated colonies were further purified by streaking them on same medium. For further selection, isolate giving fast visible iron precipitation were selected. These ten isolates were grown on casitone glycerol yeast autolysate and nutrient agar medium (Appendix I). Their iron precipitating efficiency was quantified by using 20% inoculum in citrate broth (Appendix I). Finally three best isolates were selected for further study.

Colony and morphological characterization

Colony characterization of all the thirty isolates obtained on citrate agar plate was determined in terms of shape, size, surface, elevation, margin, consistency, opacity and pigmentation of colonies. Cellular characteristics of all the thirty isolates were studied in terms of Gram's reaction, KOH test, shape, cell arrangement and motility. Similarly, colony morphology was also observed for the ten selected isolates on CGY and nutrient agar media (Appendix I). Three best isolates giving higher iron precipitation was selected for further study.

Identification

Identification of selected three isolates was done by conventional biochemical tests like carbohydrate utilization, production of oxidase, catalase and phenyl amine deaminase and hydrolysis of urea, starch and geletin and by 16S rRNA gene sequencing.

Antibiotic resistance pattern

Selected three iron precipitating isolates were studied for their antibiogram using multiple antibiotic disc containing twelve different antibiotics (PBL Bio-disc-12, India).

Polymetallic resistance of the selected isolates

Selected three isolates were tested for their resistance towards copper, chromium, cobalt and arsenic. The experiments were carried out by inoculating 1 ml of actively grown culture in test tubes

containing 20 ml of citrate broth (Appendix I) with 5, 10, 20, 40 and 80 ppm of metals like copper, chromium, cadmium and cobalt. Copper sulphate, potassium dichromate, cadmium sulphate and cobalt chloride were added as source of the respective metals. As positive control, flasks inoculated with each isolate and without addition of the metal were kept. Bacterial growth was determined by measuring optical density at 540 nm using UV visible spectrophotometer (Systronics, 119, India).

Growth profile of selected isolates

To study growth profile of three selected isolates, 5 ml of 10% (v/v) inoculum of actively growing cultures having cell load of $5.5X10^9$ cells/ml were inoculated in Erlenmeyer flasks containing 50 ml of citrate broth, tri ammonium citrate broth, and tri sodium citrate broth (Appendix I). Uninoculated flasks in the experiments served as negative control. Flasks were incubated at 30±2ºC on a rotary shaker rotating at 150 rpm. Samples were periodically removed from the filtrate obtained by filtration through Whatman filter paper no. 42 to remove the iron precipitates and growth was measured spectrophotometrically (Systronics, 119, India) in terms of optical density at 540 nm. Growth rate and generation time of the culture during the exponential growth was calculated using the following standard equation.

(1) Growth Rate :

$$K = \frac{\log_{10} Nt - \log_{10} N_0}{0.301 \times t}$$

(2) Generation time :

$$G = \frac{1}{k}$$

Where,

K = exponential growth rate constant

N_0= The population size at a certain time

N_t= The population size at subsequent time t

G = Generation time

Effect of static and shaking condition on iron bioprecipitation

Two flasks of citrate broth were inoculated with actively growing *Enterobacter* sp. having 1.1 × 10^7 cells/ml. One flask was incubated on shaker at 150 rpm and 32±2 ^oC temperature, while second one was incubated in static condition. Samples were withdrawn at 24 h intervals and iron estimation was done.

Substrate utilization profile of selected isolates in Biolog plate

The Biolog® plates, GN and GP, (Biolog Inc., USA) were used to study the utilization of specific carbon source by microorganisms from a set of 95 different carbon compounds. The Biolog® plates are designed to provide standardized biochemical tests for identifying a broad range of bacteria, which differ in the particular carbon sources in the micro plates. Biolog® plates were inoculated with 150 µl of

actively growing culture. For *Bacillus* sp., transmission was adjusted to 20%, whereas for Gram negative enteric and non enteric bacteria, transmission was adjusted to 63% and 52% respectively using the turbidometer supplied by Biolog®. For inoculum preparation the isolates were grown on BUG (Biolog® Universal Agar, Biolog® Inc., USA) medium. Plates were incubated at 35 ± 2 °C and result was recorded between 24h to 96h of incubation. Substrate utilization profile was quantified in terms of violet colour developed in Biolog® micro plates wells and used for determining diversity based on substrate utilization profile (Gupta, 2007).

Iron bioprecipitation study at different ferric ammonium citrate concentration

In iron bioprecipitation study, experiments were carried out in 250 ml Erlenmeyer flask containing 50 ml of citrate broth containing 1, 0.1 and 0.01 g/L of ferric ammonium citrate respectively. These flasks were inoculated with actively growing culture having cell load of 2 X 10^9 cells/ml of *Enterobacter* sp. or *Bacillus cereus* or *Bacillus licheniformis*. Uninoculated flask in the experimental setup was kept as negative control. The system was centrifuged at 9000 g for 15 min. The biomass was separated and aliquots were taken at regular interval of time for total iron estimation in the solution by standard phenanthroline method (Eaton et. al, 1995) (Appendix II).

Effect of inoculum size

Experiments were carried out using 250 ml Erlenmeyer flask containing 50 ml of citrate broth (Appendix 1). All the flasks were inoculated with 20, 30, 40 and 50% v/v inoculum having $2X10^9$ cells/ml of actively growing *Enterobacter* sp. or *Bacillus cereus* or *Bacillus licheniformis*. Flasks were incubated in orbital shaker at 150 rpm at 30 ±2 ^oC temperature. Uninoculated flask in the experimental setup served as negative control. The system was centrifuged at 9000 g for 15 minutes. The biomass was separated and 1 ml of supernatant was taken for total iron estimation by standard spectrophotometric Phenenthroline method (Eaton et. al, 1995) (Appendix II).

Optimization of pH

To check the pH optima for iron bioprecipitation, all the flasks with 50 ml citrate broth medium containing 1 g/L ferric ammonium citrate were adjusted at pH 3.0, 5.0 or 7.0. Flasks were inoculated with 10% (v/v) actively growing *Enterobacter* sp. having $2.2x10^9$ cells/ml. Iron was estimated periodically as described in previous part.

Results and Discussion

Sample collection

Different water and soil samples were collected for the isolation of iron precipitating bacteria from various places as detailed in Table 8.

Table 8. Collection of samples and their sites

Sr. No.	Sites	Places
1	Mine water (I)	Hutti Gold mine, Raichur dist., Karnataka, India
2	Mine water (II)	Hutti Gold mine, Raichur dist., Karnataka, India
3	Surface winze water	Hutti Gold mine, Raichur dist., Karnataka, India
4	Gas plant soil	Gujarat University, Ahmedabad, India
5	Mother Dairy	Bhat, Gandhinagar, India
6	Effluent canal water	Ekalbara, Vadodara, India
7	Tubewell water	Ekalbara, Vadodara, India
8	Well water (Luna)	Ekalbara, Vadodara, India
9	Lake water (Kankaria)	Maninagar, Ahmedabad, India

Physiochemical characteristics of water samples are shown in Table 9. All the samples collected from Hutti goldmine were clear. Sample from Mother dairy and Ekalbara were yellowish and Kankaria lake sample was green in colour. The pH value of the samples ranged from 5.4 to 9.1 with the redox potential ranging from -0.43 to 396 mV.

The samples showed variation in TDS and salinity in the range of 1.08 to 12.5 ppt and 0.6 to 2.5 ppt respectively as shown in Table 9.

Table 9. Physiochemical characterstics of water sample

Sr. No	Sample	pH	Redox Potential (mV)	Conductivity (ms)	TDS (ppt)	Salinity (ppt)
1	Mine water (I)	8.5	-0.28	3.1	1.38	1.7
2	Mine water (II)	8.5	-0.43	3.3	1.17	1.8
3	Surface winze water	9.1	-0.38	2.5	1.08	1.4
4	Gas plant soil	8.1	102	3.2	7.18	1.5
5	Inlet water - Mother Dairy	7.2	100	2.6	10.5	1.5
6	Effluent canal water	5.4	396	2.6	2.26	2.5
7	Tubewell water	6.5	360	2.4	1.34	1
8	Well water, Luna	6.9	354	2.7	1.28	1
9	Lake water, Kankaria	8	102	2.3	1.86	0.6

Isolation and screening

Total thirty different iron precipitating bacterial isolates were obtained, which formed ferric hydroxide precipitates, resulted in brown or rust coloured colonies. Their cultural characteristic is given in Table 10. Morphological characteristics of the thirty isolates obtained from citrate agar medium is given in Table 11. Out of thirty isolates, sixteen were Gram positive rods, twelve were Gram negative

rods and two were Gram positive cocci. Selected three iron precipitating cultures, isolate S_4 is a Gram negative rod, isolates GP_1 and DI_2 are Gram positive rods respectively. Cultural characteristics of ten isolates on casitone glycerol yeast autolysate and nutrient agar media are also shown in Table 11 and Table 12. Iron precipitation in liquid medium was studied and results are listed in Table 14. All the isolates showed more than 40% iron precipitation. Colony characteristics of all the ten isolates on citrate and CGY agar slant is shown in Photograph 1 and 2. Isolate which gave more than 65% iron precipitation were selected and their growth on various agar plates is shown in Photographs 3 to 8. As shown in photograph 3 and 4, colonies of *Enterobacter* sp. (S_4) and *Bacillus cereus* (GP_1) on citrate agar plate showed brown precipitation around colonies, because of ferric hydroxide deposition. On CGY medium *Enterobacter* sp. and *B. cereus* formed small to intermediate size colonies while *B. licheniformis* formed large colonies as shown in photograph 5, 6 and 7 .

Table 10. Culture characteristics of iron precipitating isolates on citrate agar medium

Isolate No	Source	Name	Shape	Size	Margin	Elevation	Surface	Consistency	Optical Characteristic	Pigmentation
1	Mother dairy, Bhat, Gandhinagar	DI_1	Round	Small	Entire	Raised	Smooth	Moist	Opaque	Brown
2	Mother dairy, Bhat, Gandhinagar	DI_2	Round	Small	Entire	Capitated	Smooth	Viscous	Opaque	Brown
3	Mother dairy, Bhat, Gandhinagar	DI_3	Round	Large	Uneven	Raised	Smooth	Moist	Opaque	Brown
4	Mother dairy, Bhat, Gandhinagar	DI_4	Irregular	Small	Undulate	Flat	Rough	Dry	Opaque	Brown
5	Surface winze water, Hutti gold mine, Raichur	S_1	Round	Small	Entire	Convex	Rough	Moist	Opaque	Brown
6	Surface winze water, Hutti gold mine, Raichur	S_2	Irregular	Medium	Uneven	Raised	Smooth	Moist	Opaque	Brown
7	Surface winze water, Hutti gold mine, Raichur	S_3	Round	Small	Entire	Flat	Smooth	Dry	Translucent	Brown
8	Surface winze water, Hutti gold mine, Raichur	S_4	Round	Medium	Entire	Raised	Smooth	Moist	Opaque	Brown
9	Minewater I, Hutti Gold, Raichur	M_1	Irregular	Large	Uneven	Flat	Smooth	Moist	Opaque	Brown
10	Minewater I, Hutti Gold, Raichur	M_2	Round	Large	Uneven	Flat	Smooth	Moist	Opaque	Brown

Isolate No	Source	Name	Shape	Size	Margin	Elevation	Surface	Consistency	Optical Characteristic	Pigmentation
11	Minewater I, Hutti Gold, Raichur	M_3	Round	Small	Entire	Raised	Smooth	Moist	Translucent	Brown
12	Minewater II, Hutti Gold, Raichur	M_{21}	Round	Medium	Entire	Flat	Smooth	Moist	Opaque	Brown
13	Minewater II, Hutti Gold, Raichur	M_{22}	Round	Small	Uneven	Flat	Rough	Moist	Translucent	Brown
14	Minewater II, Hutti Gold, Raichur	M_{23}	Round	Medium	Entire	Raised	Smooth	Dry	Opaque	Brown
15	Minewater II, Hutti Gold, Raichur	M_{24}	Irregular	Small	Entire	Flat	Smooth	Dry	Opaque	Brown
16	Minewater II, Hutti Gold, Raichur	M_{25}	Round	Large	Undulate	Convex	Smooth	Moist	Opaque	Brown
17	Gas plant soil sample, Ahmedabad	GP_1	Irregular	Large	Entire	Raised	Rough	Moist	Opaque	Brown
18	Gas plant soil sample, Ahmedabad	GP_2	Round	Medium	Entire	Flat	Smooth	Dew drop	Opaque	Brown
19	Gas plant soil sample, Ahmedabad	GP_3	Round	Small	Entire	Flat	Smooth	Moist	Opaque	Brown
20	Tubewell water, Ekalbara, Vadodara	T_1	Irregular	Small	Wavy	Raised	Smooth	Dry	Opaque	Brown
21	Tubewell water, Ekalbara, Vadodara	T_2	Round	Small	Uneven	Raised	Smooth	Moist	Opaque	Brown

Isolate No	Source	Name	Shape	Size	Margin	Elevation	Surface	Consistency	Optical Characteristic	Pigmentation
22	Tubewell water, Ekalbara, Vadodara	T_3	Round	Medium	Entire	Flat	Smooth	Moist	Opaque	Brown
23	Effluent canal water, Ekalbara, Vadodara	E_1	Round	Small	Undulate	Flat	Smooth	Moist	Transparent	Brown
24	Effluent canal water, Ekalbara, Vadodara	E_2	Irregular	Small	Uneven	Flat	Rough	Dry	Opaque	Brown
25	Effluent canal water, Ekalbara, Vadodara	E_3	Round	Medium	Entire	Flat	Smooth	Moist	Translucent	Brown
26	Well water, Luna, Ekalbara, Vadodara	L_1	Round	Medium	Undulate	Flat	Smooth	Moist	Opaque	Brown
27	Well water, Luna, Ekalbara, Vadodara	L_2	Irregular	Small	Undulate	Flat	Smooth	Moist	Opaque	Brown
28	Well water, Luna, Ekalbara, Vadodara	L_3	Round	Small	Entire	Raised	Smooth	Moist	Opaque	Brown
29	Kankria Lake, Ahmedabad	K_1	Irregular	Large	Entire	Raised	Smooth	Moist	Opaque	Brown
30	Kankria Lake, Ahmedabad	K_2	Round	Medium	Entire	Flat	Rough	Moist	Translucent	Brown

Table 11. Morphological characterization of isolates obtained from citrate agar media

Isolate Nos.	Grams reaction	Shape	Oxidase test	Catalase	KOH Test	Motility	Arrangement
DI_1	+	Rod	-	+	-	Motile	Cluster
DI_2	+	Rod	+	-	-	Motile	Single chain
DI_3	-	Rod	+	+	+	Motile	Tetrads
DI_4	-	Rod	+	-	+	Motile	Single chain
S_1	-	Rod	+	-	+	Motile	Single chain
S_2	-	Rod	+	-	+	Motile	Single chain
S_3	+	Rod	+	+	-	Motile	Single chain
S_4	-	Rod	+	-	+	Motile	Single chain
M_1	-	Rod	-	-	+	Motile	Single chain
M_2	+	Rod	+	+	-	Motile	Single chain
M_3	-	Rod	+	-	+	Motile	Single chain
M_{21}	+	Rod	+	-	-	Motile	Single chain
M_{22}	-	Rod	+	-	+	Motile	Single dyads
M_{23}	+	Cocci	-	+	-	Motile	Cluster
M_{24}	+	Rod	+	-	-	Motile	Single chain
M_{25}	+	Rod	+	-	-	Motile	Single chain
GP_1	+	Rod	-	+	-	Motile	Single chain
GP_2	+	Cocci	-	+	-	Motile	Cluster

Isolate Nos.	Grams reaction	Shape	Oxidase test	Catalase	KOH Test	Motility	Arrangement
GP_3	+	Rod	+	-	-	Motile	Single chain
T_1	-	Rod	+	-	+	Motile	Single dyads
T_2	+	Rod	+	-	-	Motile	Single chain
T_3	+	Rod	+	-	-	Motile	Single chain
E_1	+	Rod	+	-	-	Motile	Single chain
E_2	-	Rod	+	-	+	Motile	Single dyads
E_3	+	Rod	+	-	-	Motile	Single chain
L_1	+	Rod	+	-	-	Motile	Single chain
L_2	-	Rod	+	-	+	Motile	Single dyads
L_3	+	Rod	+	-	-	Motile	Single chain
K_1	+	Rod	+	-	-	Motile	Single chain
K_2	-	Rod	+	-	+	Motile	Single dyads

Table 12. Culture characteristics of iron precipitating isolates on CGY medium

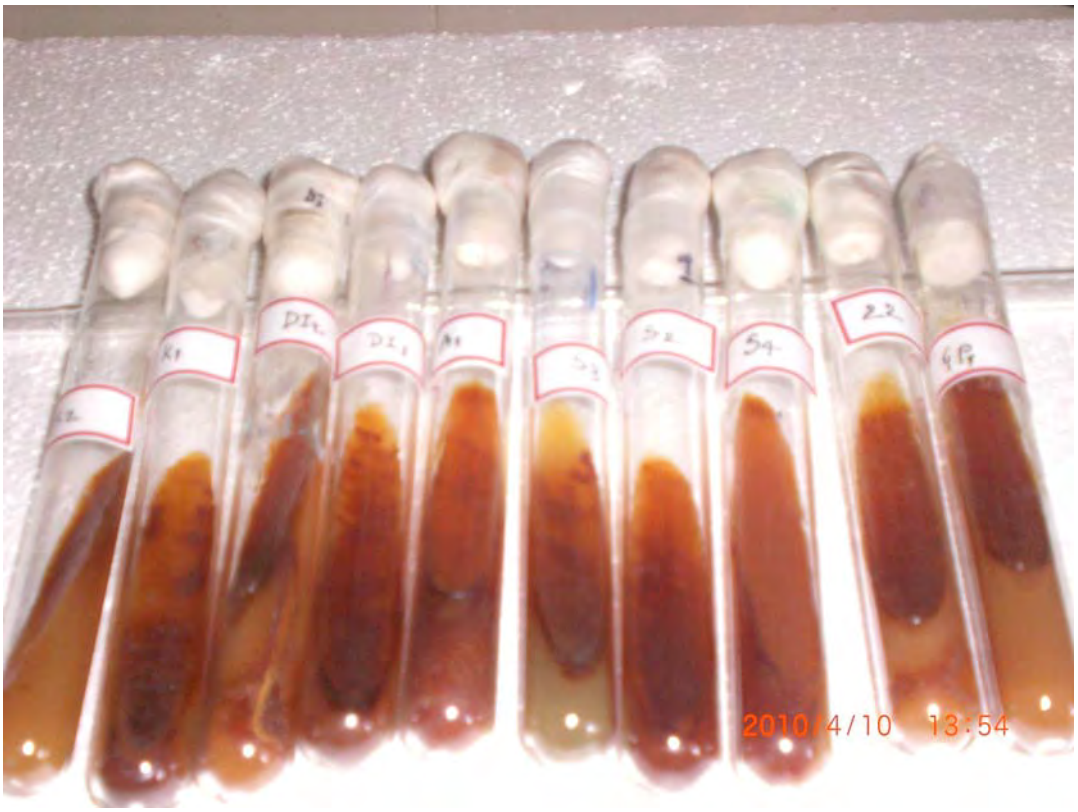
Isolate No	Name	Shape	Size	Margin	Elevation	Surface	Consistency	Optical Characteristic
1	DI_2	Round	Small	Undulate	Raised	Smooth	Moist	Opaque
2	M_{21}	Round	Small	Entire	Raised	Smooth	Moist	Opaque
3	T_1	Punctiform	pinpoint	Entire	Flat	Echinate	Dewdrop	Translucent
4	E_1	Round	Small	Entire	Raised	Smooth	Dry	Opaque
5	M_1	Round	Medium	Undulate	Raised	Smooth	Moist	Translucent
6	L_2	Round	Small	Entire	Convex	Echinate	Moist	Opaque
7	S_4	Round	Medium	Entire	Flat	Echinate	Moist	Opaque
8	K_1	Round	Medium	Entire	Flat	Smooth	Moist	Opaque
9	M_2	Irregular	Medium	Undulate	Flat	Rough	Dry	Opaque
10	GP_1	Round	Big	Entire	Raised	Smooth	Moist	Opaque

Table 13. Culture characteristics of iron precipitating isolates on nutrient agar medium

Isolate No	Name	Shape	Size	Margin	Elevation	Surface	Consistency	Optical Characteristic	Pigmentation
1	DI_2	Round	Medium	Entire	Effused	Smooth	Butyrous	Opaque	Nil
2	M_{21}	Round	Medium	Entire	Effused	Smooth	Moist	Opaque	Nil
3	T_1	Irregular	Medium	Entire	Flat	Rough	Moist	Transparent	Nil
4	E_1	Round	Small	Entire	Raised	Smooth	Moist	Resinous	Nil
5	M_1	Punctiform	Pinpoint	Entire	Flat	Glistening	Dewdrop	Translucent	Nil
6	L_2	Round	Medium	Undulate	Flat	Smooth	Butyrous	Resinous	Nil
7	S_4	Round	Medium	Entire	Flat	Smooth	Moist	Opaque	Nil
8	K_1	Round	Medium	Undulate	Flat	Smooth	Moist	Opaque	Nil
9	M_2	Punctiform	Small	Entire	Flat	Rough	Dry	Opaque	Nil
10	GP_1	Round	Medium	Entire	Raised	Smooth	Moist	Opaque	Nil

Table 14. Iron precipitation by different isolates at 20% (v/v) inoculum size

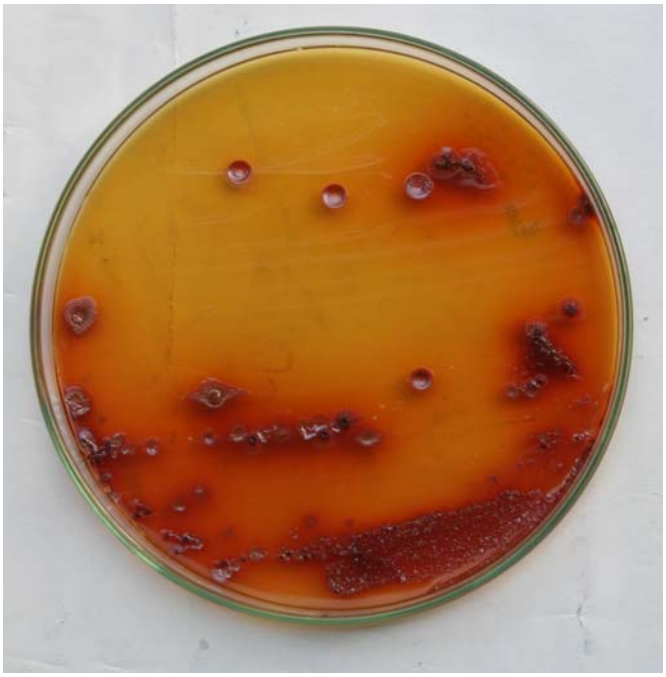
Isolate No	Iron precipitation (%)
M_{21}	53.34
T_1	43.75
GP_1	70
K_1	58.75
DI_2	67
E_1	46.6
S_4	93
M_1	60.5
L_2	50
M_2	52



Photograph 1. Growth of different isolates on citrate agar medium

Photograph 2. Growth of different isolates on CGY medium

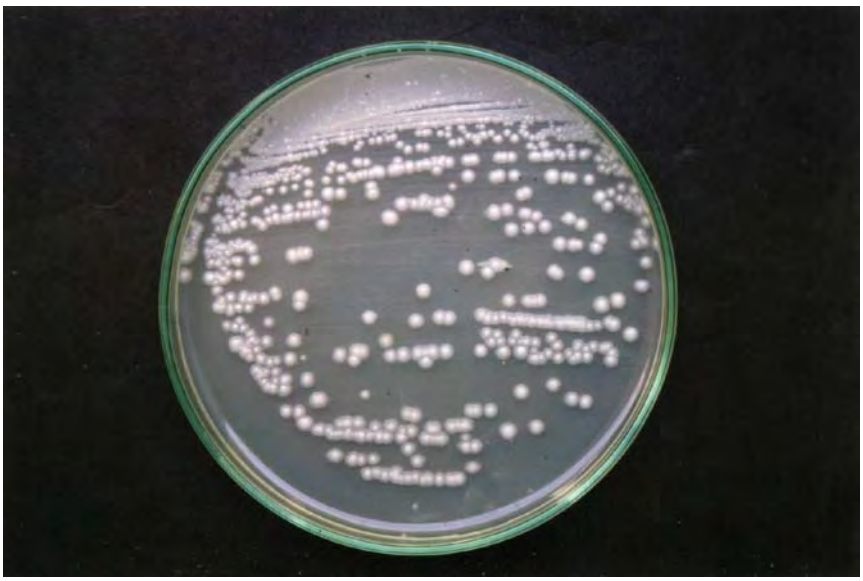
Photograph 3. Growth of *Enterobacter* sp. (S_4) on citrate agar medium



Photograph 4. Growth of *Bacillus cereus* (GP_1) on citrate agar medium

Photograph 5. Growth of *Enterobacter* sp. (S_4) on CGY medium

Photograph 6. Growth of *Bacillus licheniformis* (DI_2) on CGY medium



Photograph 7. Growth of *Bacillus cereus* (GP_1) on CGY medium

Photograph 8. Growth of *Enterobacter* sp. (S_4) on nutrient agar medium

Biochemical characteristics of isolates

Biochemical tests of selected three iron precipitating isolates is given in Table 15.

Table 15. Biochemical tests of selected isolates

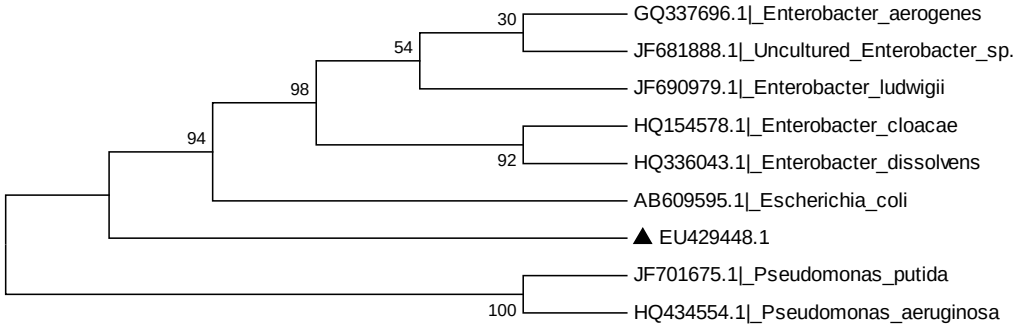
Biochemical test	S_4		GP_1		DI_2	
Sugar Fermentation	**Acid**	**Gas**	**Acid**	**Gas**	**Acid**	**Gas**
Glucose	+++	+++	+++	-	+	-
Sucrose	+++	+++	+	-	+	-
Lactose	++	-	-	-	-	-
Maltose	++	++	+++	-	+	+
Mannitol	+++	++	-	+	+	-
Fructose	+++	+++	+	+	+	+
Sorbitol	+	-	+	+	+	+
Galactase	+	+	+	-	+	-
Xylose	++	++	-	+	-	+
Nitrate Reduction	+		+		+	
Methyl Red	-		-		-	
Vogus Proskaur	+		+		+	
Phenyl alanine Deamination	-		-		-	
Urea Hydrolysis	+		+		-	
Oxidative Fermentation						
a) With Oil	-		-		-	
b) Without Oil	+		+		-	
Simmons Citrate	+		+		+	
Starch Hydrolysis	+		+		+	
Gelatin Hydrolysis	+		+		+	
KOH	+		-		-	

Biochemical test	**S_4**	**GP_1**	**DI_2**
Oxidase	+	+	+
Vancomycin	-	+	+
Triple Sugar Iron			
a)Butt	Acidic	Alkaline	Acidic
b)Slant	Acidic	Alkaline	Alkaline
c)Gas	+	-	-
d)H_2S	-	-	-
SIM			
a) Motility	+	+	+
b) H_2S Protection	-	-	-
c) Indole	-	-	-
MacConkey	Large, Smooth, irregular, edged, convex, mucoid, pink coloured colonies	Large, undulate, opaque, lactose non fermentor colonies	Small, entire, lactose non fermentor colonies
EMB Agar	Large, round, smooth, pinkish, non nucliated colonies	Large, entire, smooth, white colonies	Small, entire, round, creamish colonies

Identification

The three iron precipitating cultures were identified as *Enterobacter cloacae* (S_4), *Bacillus lichiniformis* (DI_2) and as *Bacillus cereus* (GP_1) by 16S rRNA gene sequencing. The sequences of *Enterobacter cloacae* and *Bacillus licheniformis* are deposited in GenBank under the accession no. EU429448 and EU429447. The phylogenetic trees are shown in Figure No. 8.

A)



B)

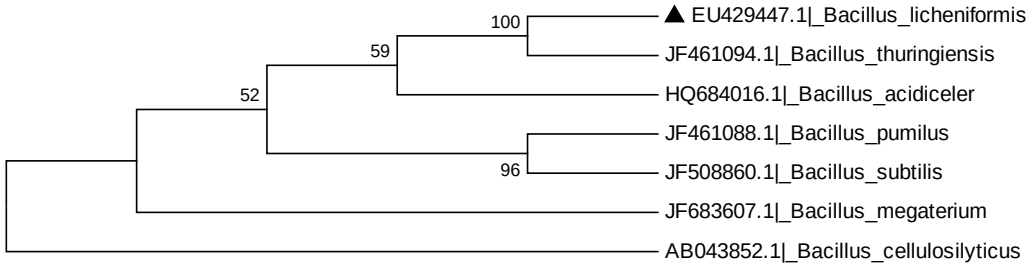


Figure 8. Phylogenetic tree A). *Enterobacter cloacae* (EU429448) and B). *Bacillus licheniformis* (EU429447)

Antibiotics sensitivity study

Antibiogram result of *Enterobacter* sp. is shown in Table 16. This isolates showed resistant to antibiotics piperacillin and gatifloxacin. It showed variable level of sensitivity to rest of the antibiotics studied. It gave largest zone of inhibition with chloramphenicol. *Bacillus cereus* is resistant to cefotaxime and cloxacillin. *Bacillus licheniformis* resistant to cefotaxime and ofloxcin are given in Table 17.

Table 16. Antibiogram of *Enterobacter* sp.

Sr. No.	Antibiotics	Strength (µg/disc)	Inhibition zone size (mm)
1	Ampicillin	20	26
2	Cotrimoxazole	25	20
3	Cefotaxime	30	24
4	Piperacillin	100	Resistant
5	Chloramphenicol	30	28
6	Ciprofloxacin	5	25
7	Ceftizexime	30	22
8	Tetracycline	30	21
9	Ofloxacin	5	23
10	Gentamicin	10	15
11	Amikacin	30	14
12	Gatifloxacin	10	Resistant

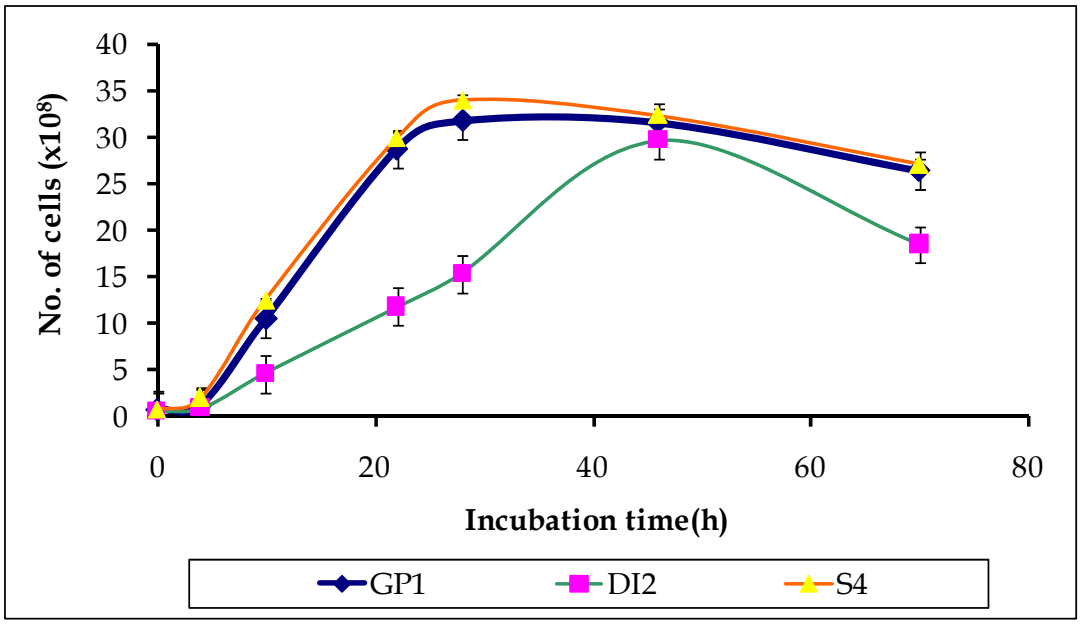
Table 17. Antibiogram of *Bacillus*

Sr. No.	Antibiotics	Strength (µg/disc)	Inhibition zone size (mm)	
			Bacillus cereus	*Bacillus licheniformis*
1	Ampicillin	20	23	20
2	Cephalexin	30	12	18
3	Tetracycline	30	18	14
4	Cefotaxime	30	Resistant	Resistant
5	Ciprofloxacin	5	25	25
6	Pefloxacin	10	28	22
7	Ofloxacin	5	18	Resistant
8	Cloxacillin	1	Resistant	15
9	Roxythromycin	15	19	12
10	Lincomycin	2	28	24
11	Gentamicin	10	24	14
12	Cotrimoxazole	25	22	20

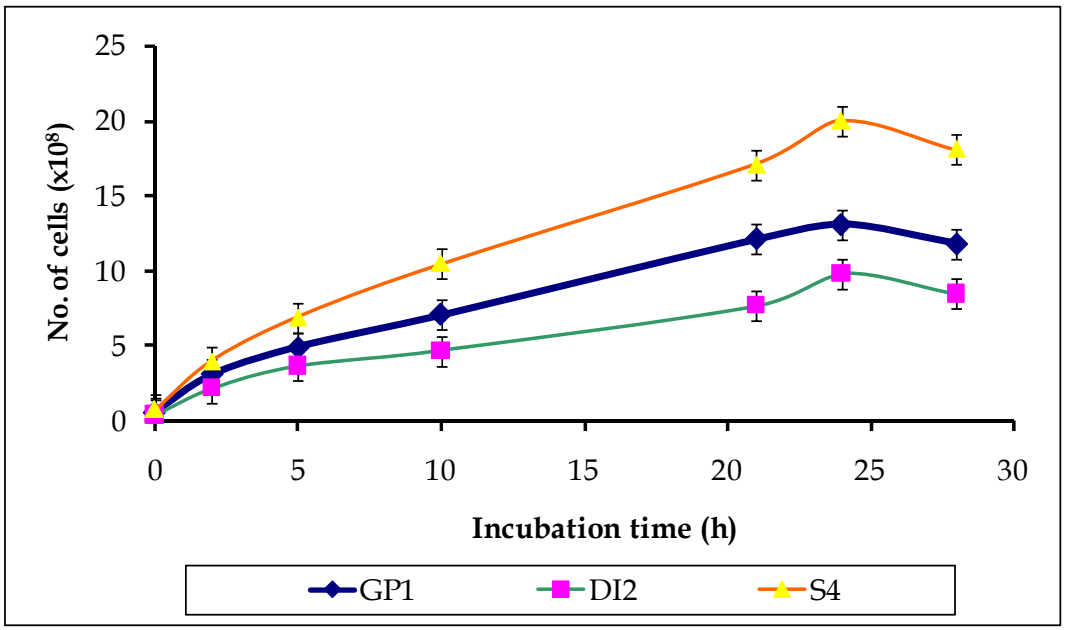
Growth profile study

Growth profile of selected three isolates was compared in TSC (Tri-sodium citrate) broth, TAC (Tri ammonium citrate) broth and citrate broth. In TSC broth and citrate broth *Enterobacter* sp. showed the fastest growth as compared to *Bacillus cereus* and *Bacillus licheniformis.* Results are depicted in Graph 1 and 2. The generation time of *Bacillus cereus, Bacillus licheniformis* and *Enterobacter* sp. was 5.1, 8.9 and 2.28 h in TSC broth and 4.6, 10.2 and 3.8 h in citrate broth respectively. As can be seen from Graph 3 in TAC broth *Bacillus cereus* showed faster growth as compared to other two isolates studied. The generation time of *Bacillus cereus, Bacillus licheniformis* and *Enterobacter* sp. in TAC broth was 4.41, 5.2 and 4.91 h respectively. *Enterobacter* sp. showed good growth and higher iron precipitation under shaking condition than static condition as shown in Graph 4.

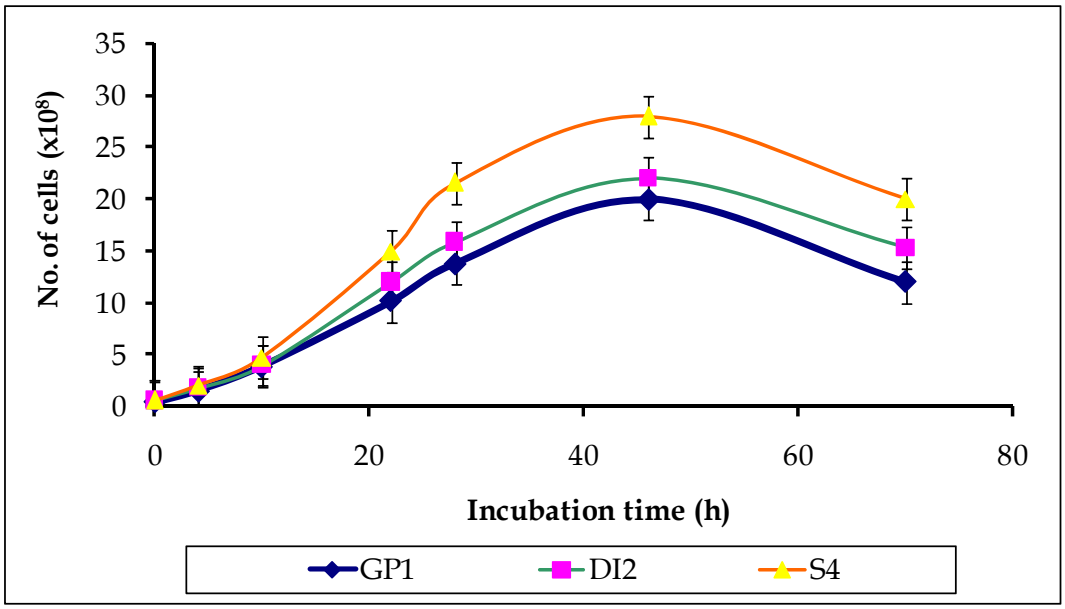
Graph 1. Growth phase of *Enterobacter* sp. (S_4), *Bacillus cereus* (GP_1) and *Bacillus licheniformis* (DI_2) in TSC broth



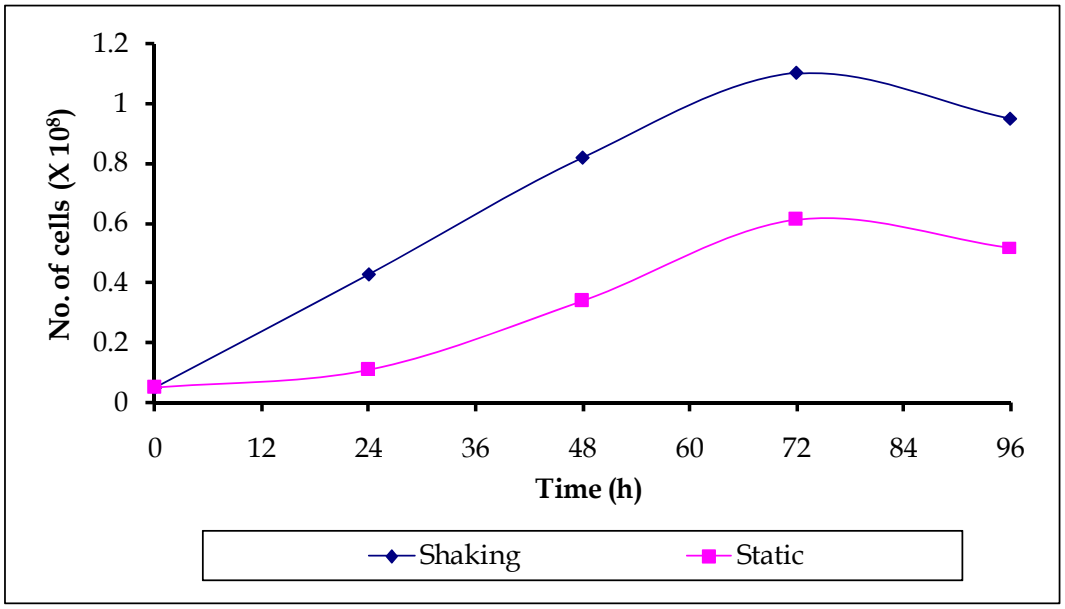
Graph 2. Growth phase of *Enterobacter* sp. (S_4), *Bacillus cereus* (GP_1) and *Bacillus licheniformis* (DI_2) in citrate broth



Graph 3. Growth phase of *Enterobacter* sp. (S_4), *Bacillus cereus* (GP_1) and *Bacillus licheniformis* (DI_2) in TAC broth



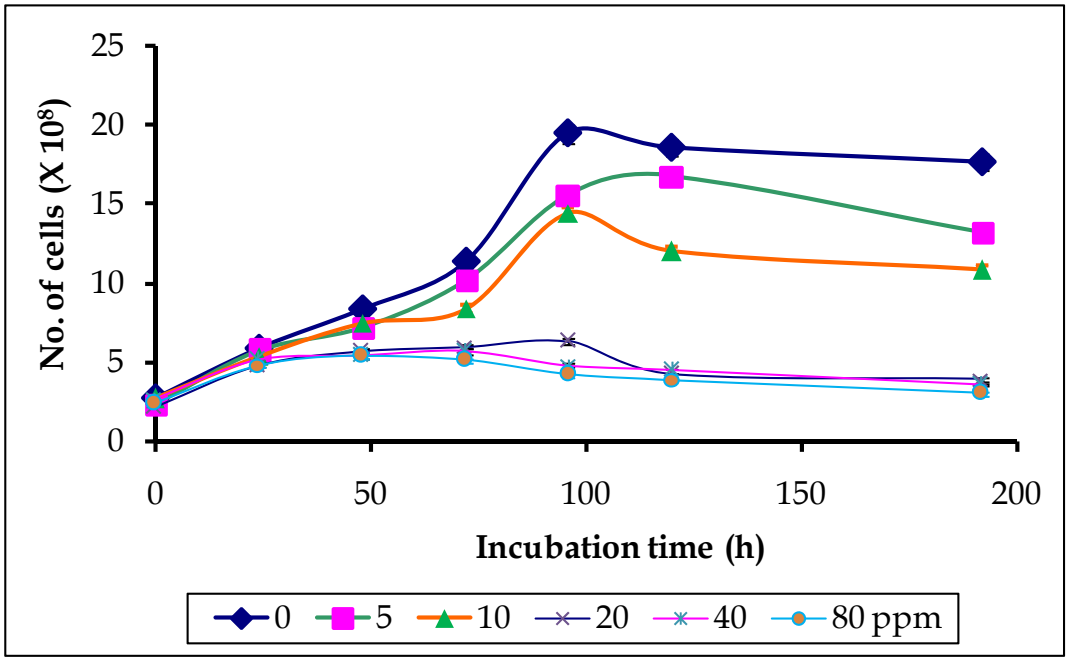
Graph 4. Growth profile of *Enterobacter* sp. (S_4) in shaking and static conditions



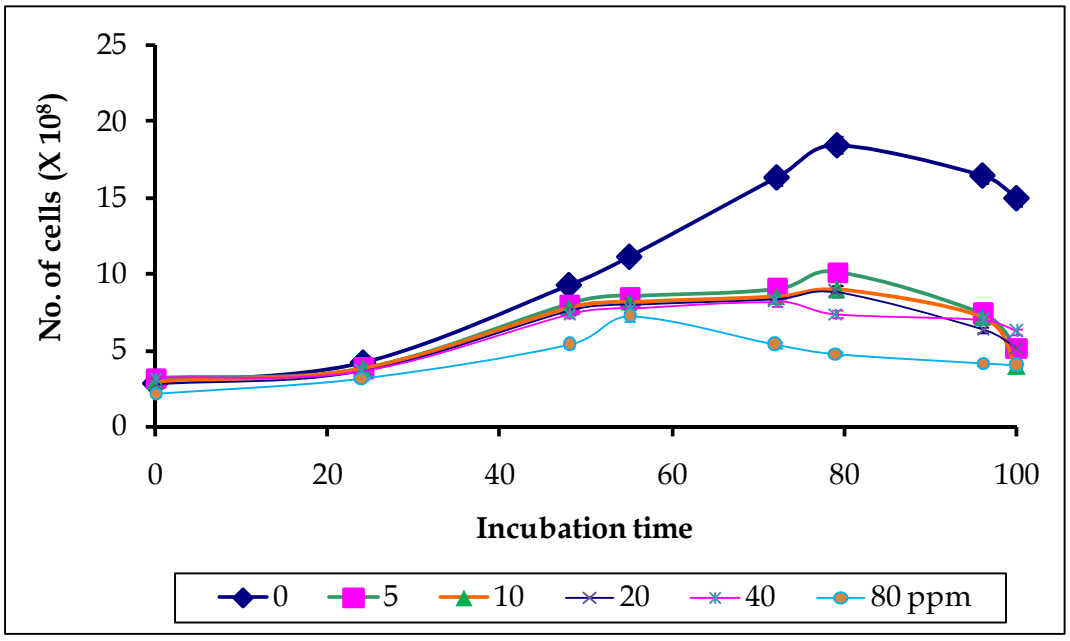
Polymetallic resistance of selected isolates

All the three isolates were studied for their metal and metalloid resistance and were found to be resistant up to 80 ppm of copper, arsenic, cobalt and chromium. *Enterobacter* sp. and *Bacillus cereus* grew well up to 10 ppm of arsenic, where as *Bacillus licheniformis* was resistant up to 5 ppm of arsenic as shown in Graphs 5, 9 and 14. *Enterobacter* sp. showed good growth and resistance even upto 80 ppm of copper as compared to other two isolates in Graphs (7, 11 and 13). As can be seen from the results shown in Graphs 6, 10 and 15 *Bacillus licheniformis* and *Bacillus cereus* showed resistance up to 5ppm of cobalt, where as *Enterobacter* sp. showed resistance up to 10 ppm of cobalt. *Enterobacter* sp., *Bacillus cereus* and *Bacillus licheniformis* showed the lowest resistance towards chromium as shown in Graphs 8, 12 and 16.

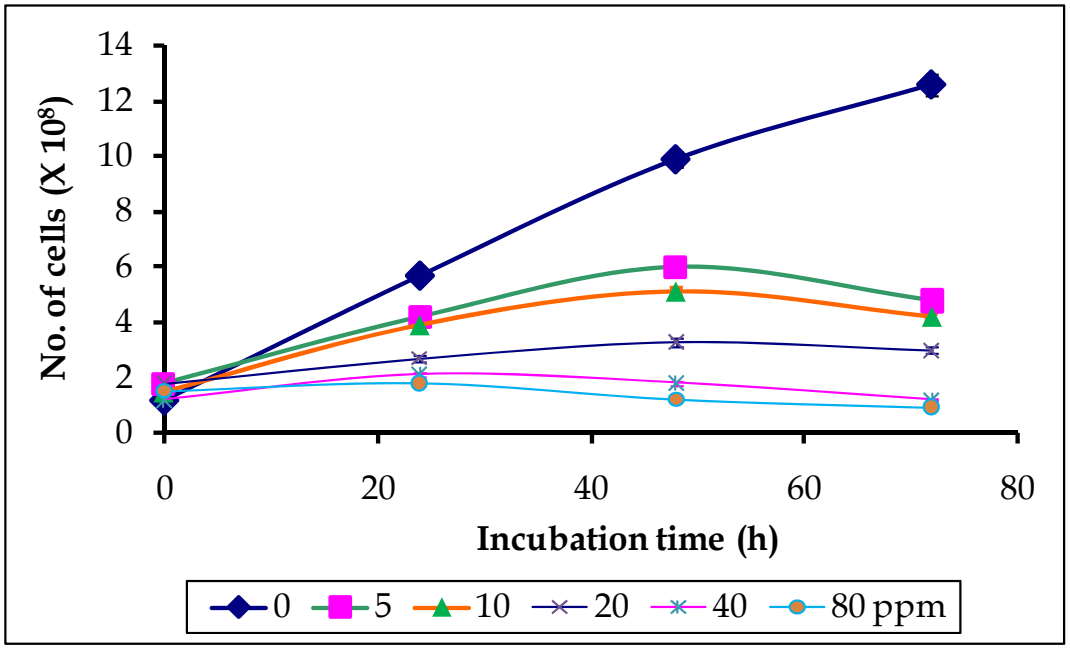
Graph 5. Growth profile of *Bacillus cereus* (GP_1) in the presence of various arsenite concentrations



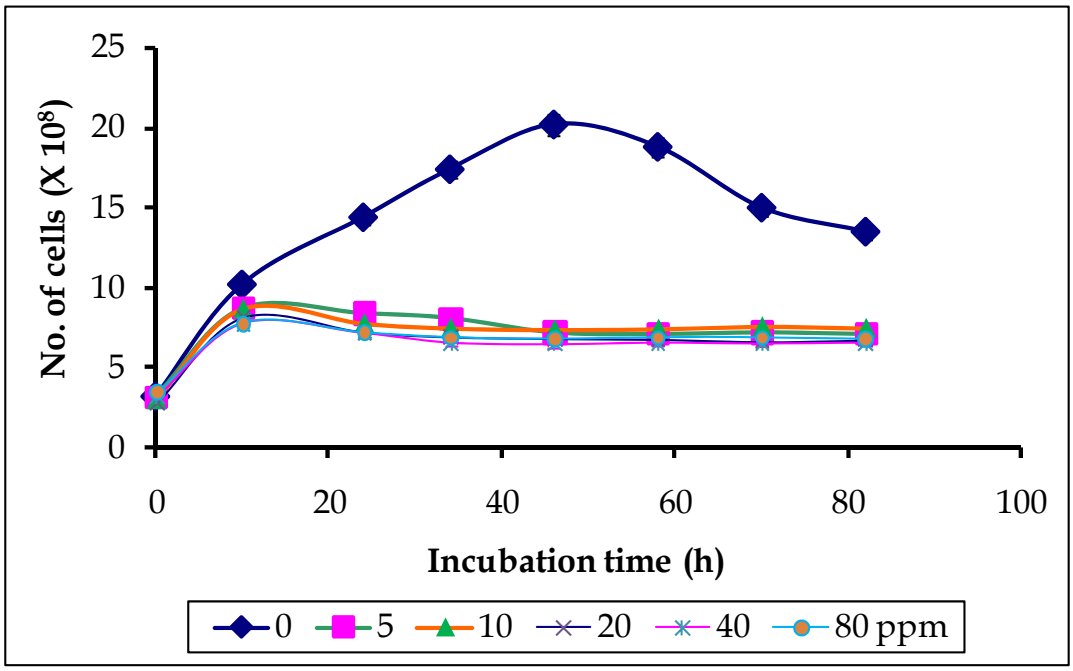
Graph 6. Growth profile of *Bacillus cereus* (GP_1) in the presence of cobalt



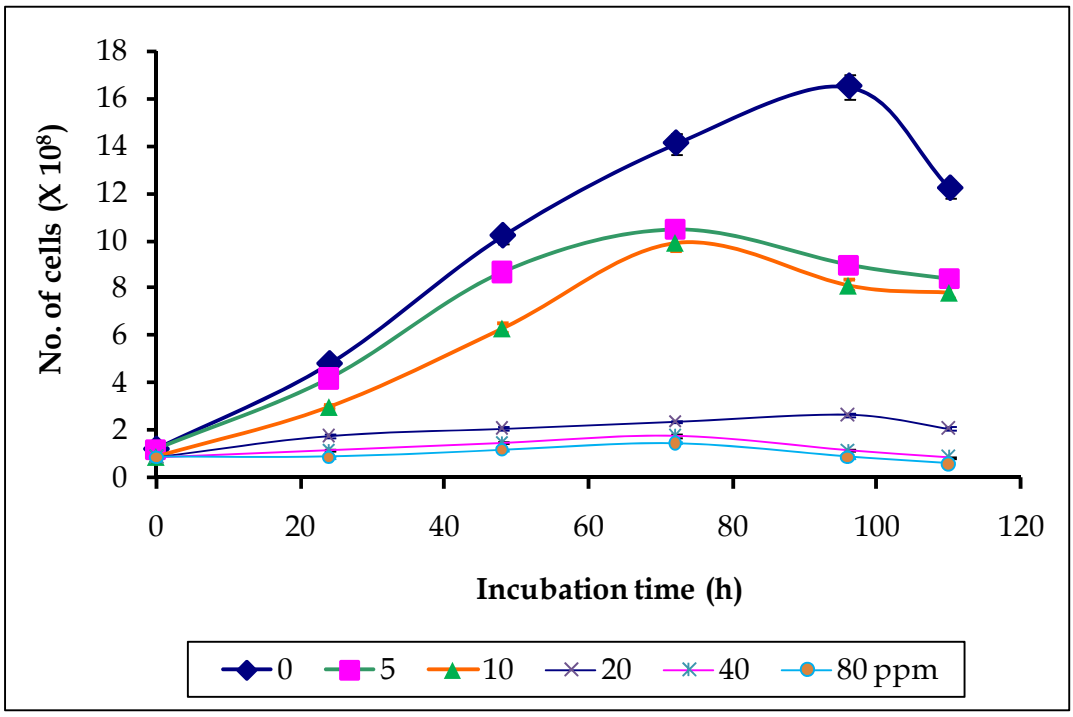
Graph 7. Growth profile of *Bacillus cereus* (GP_1) in the presence of copper



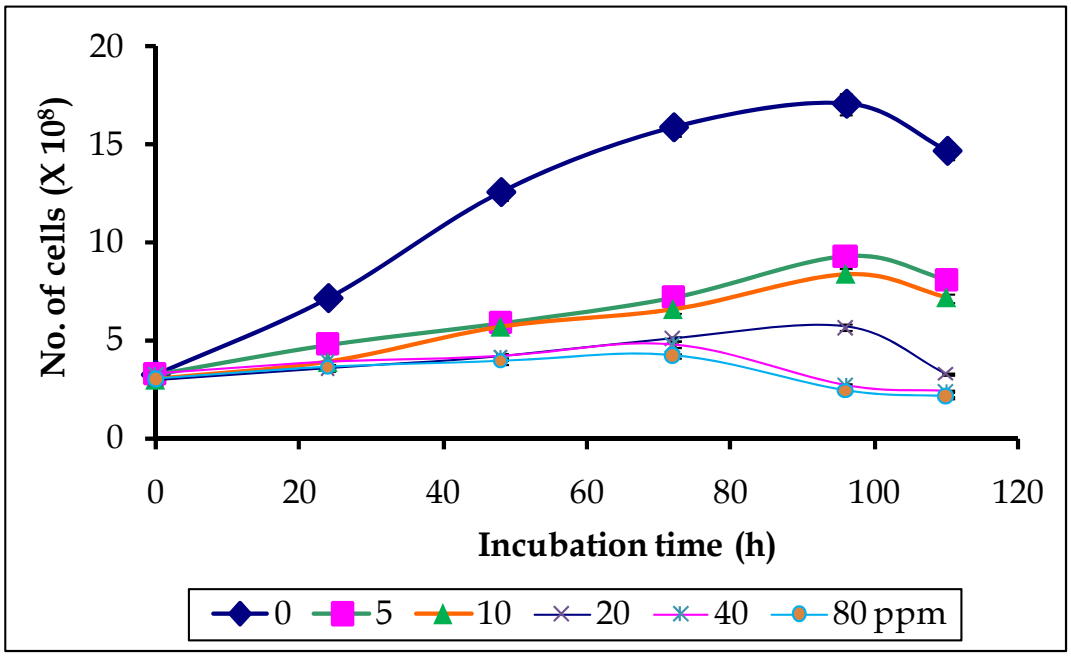
Graph 8. Growth profile of *Bacillus cereus* (GP_1) in the presence of chromium



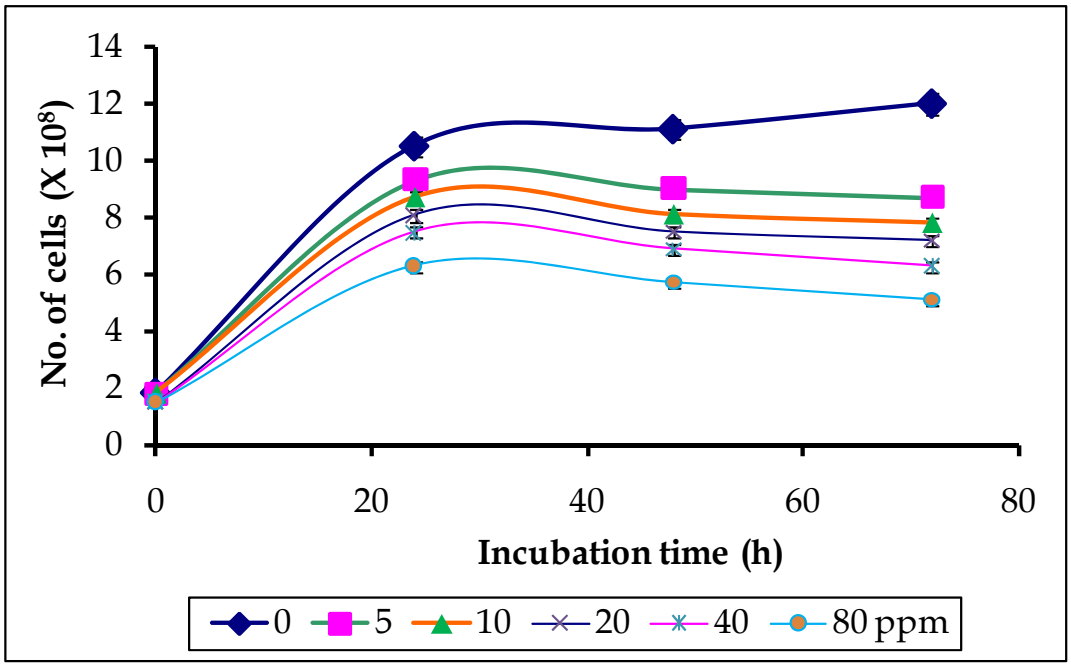
Graph 9. Growth profile of *Enterobacter* sp. (S_4) in the presence of arsenite



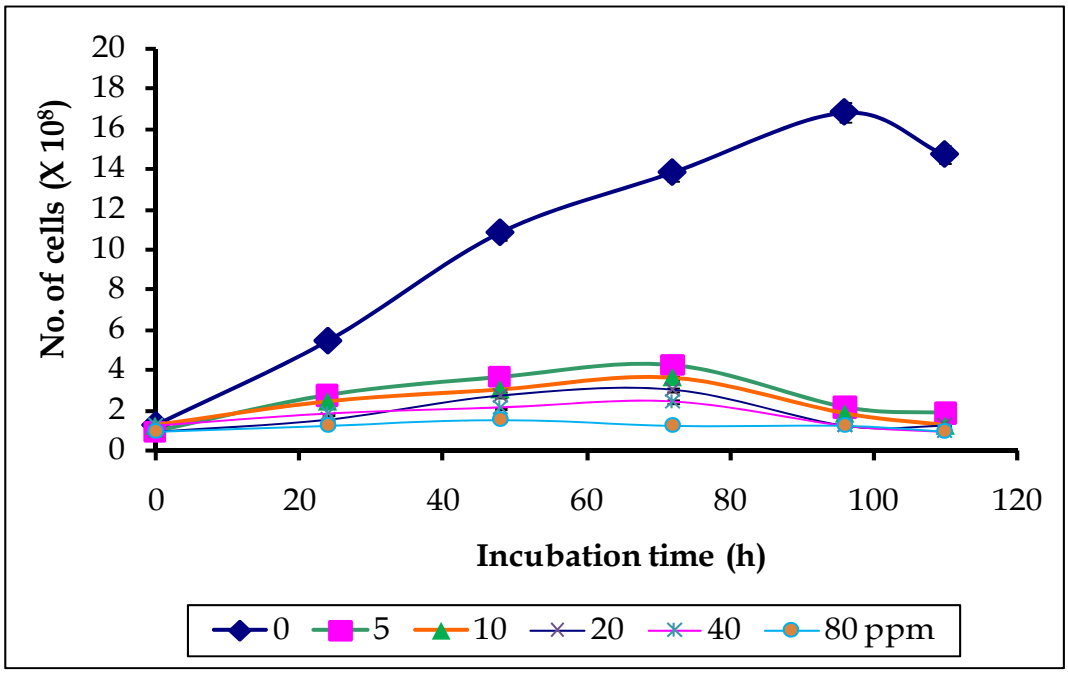
Graph 10. Growth profile of *Enterobacter* sp. (S_4) in the presence of cobalt



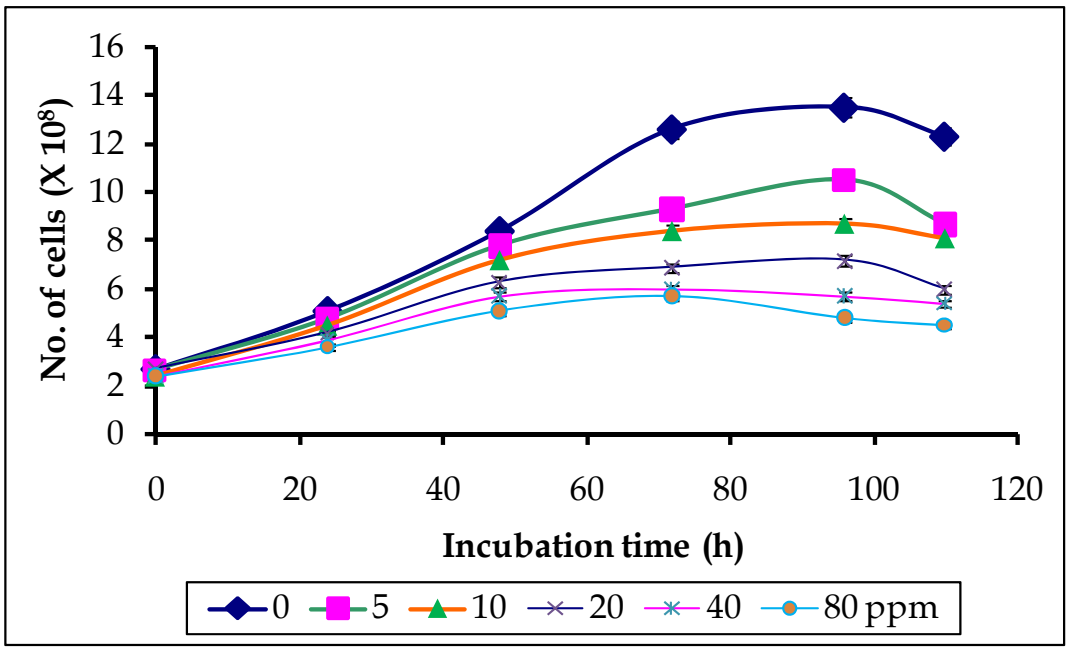
Graph 11. Growth profile of *Enterobacter* sp. (S_4) in the presence of copper



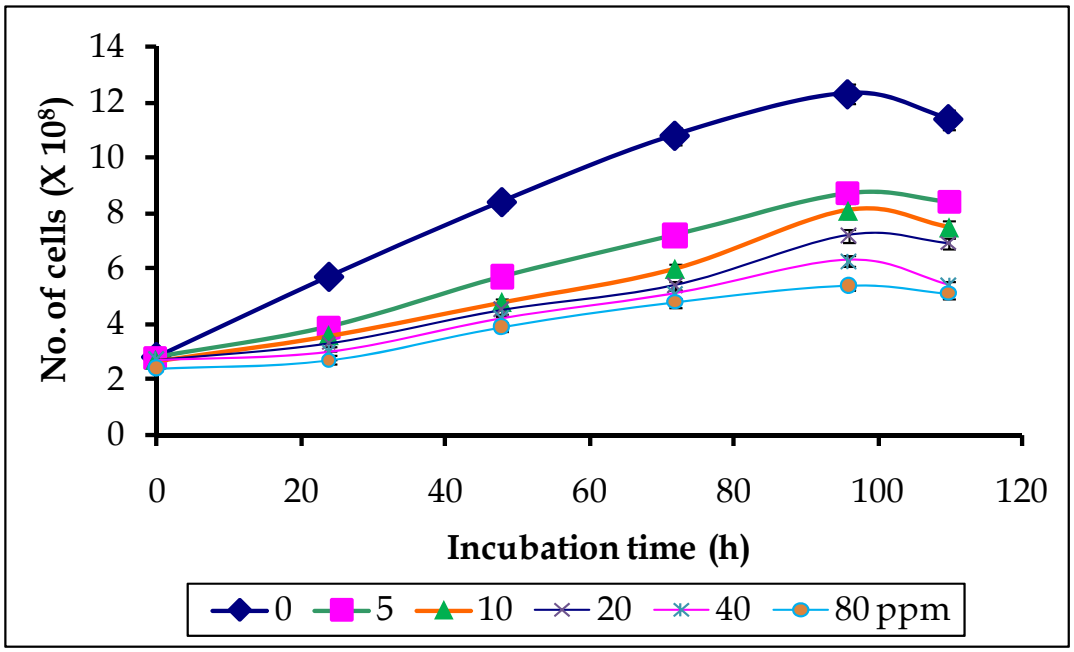
Graph 12. Growth profile of *Enterobacter* sp. (S_4) in the presence of chromium



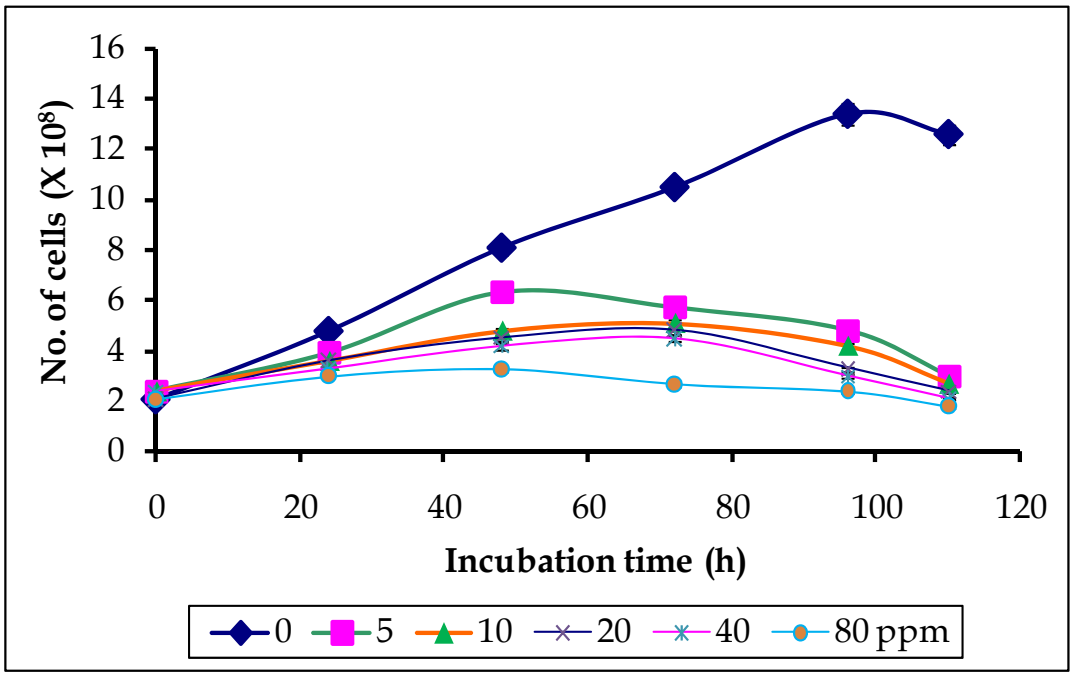
Graph 13. Growth profile of *Bacillus licheniformis* (DI_2) in the presence of copper



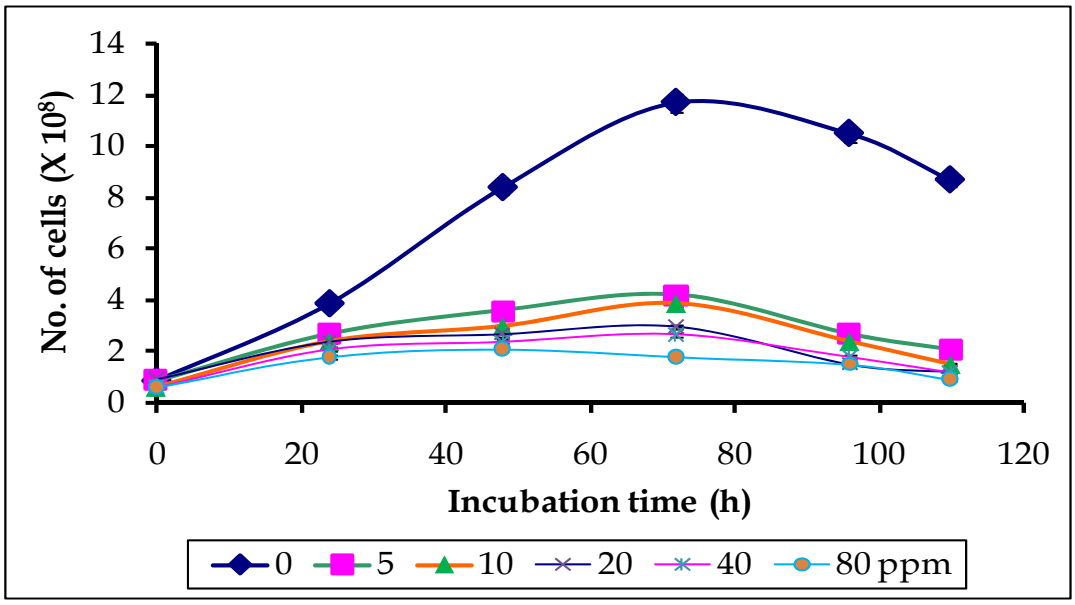
Graph 14. Growth profile of *Bacillus licheniformis* (DI_2) in the presence of arsenite



Graph 15. Growth profile of *Bacillus licheniformis* (DI_2) in the presence of cobalt



Graph 16. Growth profile of *Bacillus licheniformis* (DI_2) in the presence of chromium



Substrate utilization profile of the selected isolates and their identification by Biolog ® plates.

List of substrates in Gram positive and Gram negative Biolog® plates are given in Table 18 and 19. When substrate utilization pattern of individual isolate was studied, it showed considerable diversity in terms of substrate they metabolized. When substrate utilization profile of Gram negative isolate was studied, it was observed that isolate S_4 (*Enterobacter* sp.) utilized maximum number of substrates from all groups and total number of substrates utilized came out to be 61. Isolate S_4 utilized N-acetyl-D-glucosamine, α D-glucose, maltose, sucrose, citric acid, L-leucine, L-alanine, L-proline glycogen, dextrin, glycerol, D-glucose-6-phosphate, succinic acid in 24 h of incubation. Gentibiose, D-fructose, D-mannose, D-trehalose, D-raffinose, adonitol, D-galactose, D-gluconic acid, α hydroxyl

Table 18. List of substrates in Gram positive Biolog® plate

Carbohydrates	Carbohydrates	Polymers	Phosphrylate chemical
α –D glucose	turanose	α –cyclodextrin	D-L- α -glycerol phosphate
α –D lactose	xylilol	Dextrin	α –D-glucose- 1 - phosphate
α –Methyl D – glucoside	3 Methyl D-glucose	β – Cyclodextrin	D-glucose-6- phosphate
N acetyl D glutamic acid	sucrose	Glycogen	adenosine-5- monophosphate
N aceyl D glucasamine	mannan	tween 40	thymidine-5- monophosphate
Arbutin	inulin	tween 80	uridine-5- monophosphote
D – cellobiose	palatinose	Lactoamide	D-fructose-6- phosphate
D –arbitol	Salicin	L –lactic acid	**Amines/ Amides**
D –fructose	Sedoheptulose	D – alanine	phenyl-ethylamide
D-galactose	Stachyose	Glycyl – L –asparatic acid	2-amino ethnol
D –mannitol	N –acetyl D- glucosamine	Glycyl –L- glutamic acid	putriscine
D –mannose	N –acetyl D – Mannosamine	L –alanine	alananin amide
D –melezitose	N –acetyl D –galactoside	D –malic acid	glucuron amide
	α methyl D galactoside		

D-melebiose	B –Methyl D –galactoside	L – malic acid
D –raffinose	**Carboxylic acid**	L –alanyl glycine
D –psicose	Aetic acid	L – glutamic acid
D –sorbitol	α hydroxyl butyric acid	L- asparagines
D –trehlose	β - hydroxy butyric acid	L –pyroglutamic acid
D –taratose	γ- hydroxy butyric acid	**Alcohols**
L –arabinose	β - hydroxyphenyl acetic acid	2,3 butanediol
L –fucose	α –ketoglutaric acid	Glycerol
L –rhamnose	α –keto valeric acid	**Aromatic compound**
D –ribose	pyruvic acid	Inosine
Lactulose	D- galactouronic acid	Thymidine
m –Inositol	D – gluconic acid	Uridine
amygdolin	L –lactic acid	**Esters**
gentibiose	propionic acid	pyruvic acid methylester
Maltose	succinic acid	succinic acid mono methyl ester
maltotriose		D – lactic acid methyl ester

Table 19. List of substrate in Gram negative Biolog® plate

Carbohydrates	Carboxylic acid	Amino acids	Polymers
N-acetyl-D-galactosamine	acetic acid	D-alanine	glycogen
N-acetyl-D-glucosamine	Cis-Acotonic acid	L-alanine	α cyclodextrin
Adonitol	Citric acid	L-alanyl-glycine	dextrin
L-arabinose	Formic acid	L-asparagine	tween 80
D-cellobiose	D-galactonic acid	L-aspartic acid	tween 40
L-erythritol	lactone	L-glutamic acid	**Phosphorylated chemical**
D-fucose	D-glucornic acid	Glycyl-L-glutamic acid	D-L-α-glycerol phosphate
D-galactose	D-glucosaminic acid	Glycyl-L-aspartic acid	α –D-glucose-1-phosphate
Gentiobiose	D-glucoronic acid	L-histidine	D-glucose-6-phosphate
α D-glucose	α -hydroxl butyric acid	Hydroxy –L-proline	**Esters**
m-Inositol	β-hydroxybutyric acid	L-luecine	pyruvic acid methyl ester
α lactose	γ-hydroxy butyric acid	L-ornithine	succinic acid monomethyl ester
Lactulose	p-hydroxyphenylacetic acid	L-phenylalanine	**Brominated chemicals**
Maltose	itaconic acid	L-proline	bromo succinic acid
D-mannitol	α -ketobutyric acid	L-pyroglutamic acid	**Aromatic compounds**
D-raffinose	α -Ketoglutaric acid	D-serine	iInosine

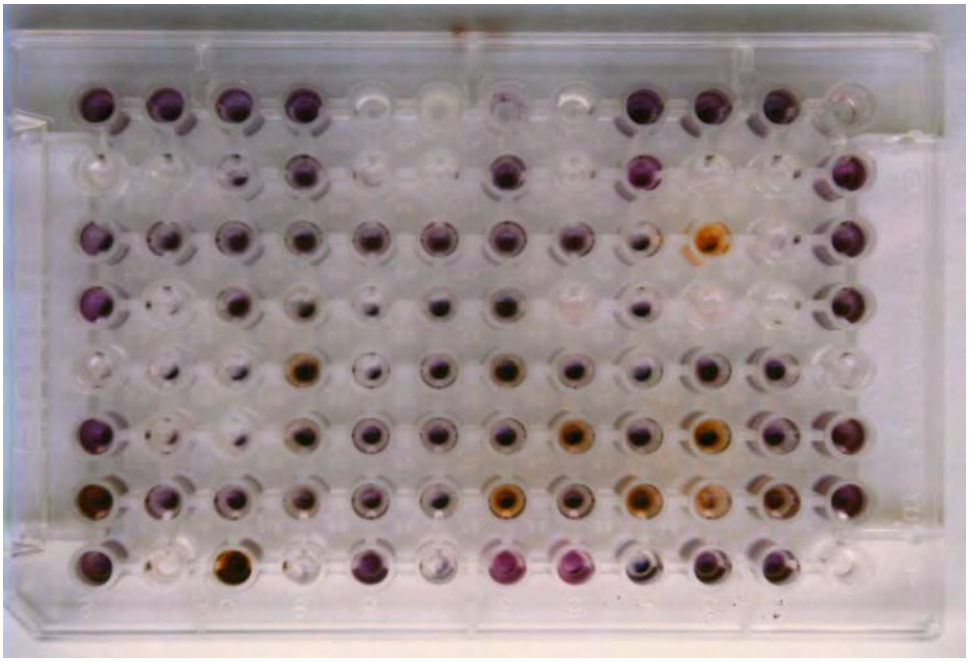
L-rhamnose	D, L-Lactic acid	L-serine	uridine
D-sorbitol	malonic acid	L-threonine	thymidine
Sucrose	propionic acid	D,L-carnitine	urocanic acid
D-trehalose	quinic acid	y-aminobutyric acid	glucuronamide
Turanose	D-saccharic acid	**Amines/Amides**	
Xylitol	sebacic acid	Alaninamide	
D-fructose	succinic acid	succinamic acid	
D-arabitol	α -ketovaleric acid	L-phenylethylamine	
D-mannose	**Alcohols**	2 –aminoethanol	
D-melibiose	2,3-butanediol	Putriscine	
D-psicose	glycerol		
βMethyl D-glucoside			

butyric acid, quinic acid, malonic acid, propionic acid, L- aspartic acid, L-phenylalanine, L-histidine, tween 40, α -D-glucose-1-phosphate, succinic acid monomethyl ester, alaninamide, L-Phenylethylamine, D-glucose-6-phosphate in 48 h of incubation. Inosine, uridine, putriscine, hydroxy-L-proline, L-glutamic acid, L-alanyl-glycine, α ketobutyric acid, itaconic acid, D-Glucornic acid,propionic acid, p-hydroxy phenylacetic acid, D-galacturonic acid, thymidine, D-melibiose, turanose, D-sorbitol, D-galacturonic acid, α-ketobutyric acid, glycyl-L-asparatic acid, L-leucine, α-cyclodextrin, lactulose, α-lactose in 96 h of incubation. 2,3 – butanediol, bromosuccinic acid, pyruvic acid methyl ester, glucuronamide and acetic acid were not utilized by the isolate even after 96 h of incubation.

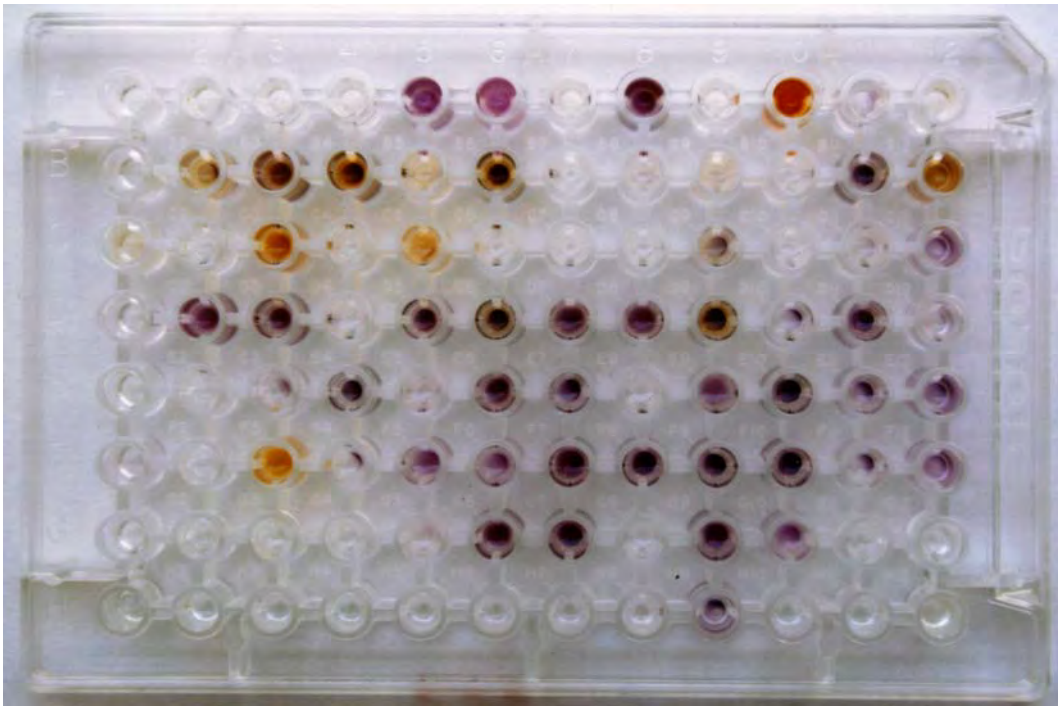
In Gram positive isolates, isolate GP_1 (*Bacillus cereus*) and isolate DI_2 (*Bacillus licheniformis*) were able to utilize 50 and 12 substrates respectively, out of 95 substrates studied. Isolate GP_1 utilized carbohydrates like α D-glucose, α methyl-D-glucoside, sucrose, D-fructose, D-mannitol, D-sorbitol, N-acetyl-D-mannosamine, N-acetyl–D-glucosamine, L-fucose, D-arbitol, D-melebiose, turanose and phosphorylated chemicals like D-glucose-6-phosphate, D-fructose-6- phosphate, propionic acid, succinic acid,glycyl-L-glutamic acid in 24 h of incubation. D-ribose, D-raffinose, gentibiose, arbutin, maltose, α- D-lactose-D-pscicose, dextrin, glycogen, D-alanine, L-asparagine, L-glutamic acid, α-cyclodextrin, β cyclodetrin, tween 40, L-lactic acid, glycyl-L-asparatic acid, putriscine, alanin amide, succinic acid monomethyl ester, D-lactic acid methyl ester, 2-amino ethanol, 2,3 butanediol. Inulin, mannon, salicin, sedoheptulose, L-

pyroglutamic acid, glycerol in 48 h of incubation. Pyruvic acid methyl ester, glucoronamide, adenosine-5-monophosphate, thymidine 5 monophosphate need prolonged incubation time to utilized. Acetic acid, α ketovaleric acid, α-ketobutyric acid and aromatic compounds Inosine, thymidine and uridine were not utilized.

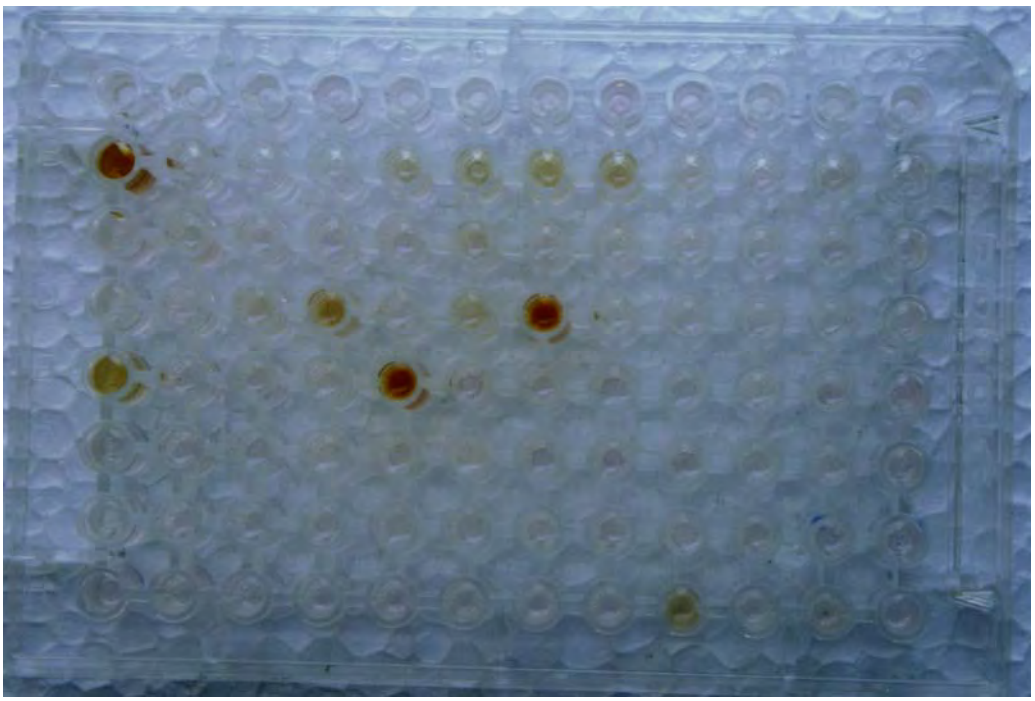
Isolate DI_2 was observed to utilize minimum number of substrates from each category and unable to utilize any substrate from amine, amides, aromatic compounds and alcohol groups. D-sorbitol, D-fructose, sucrose and α –D-glucose in 24 h of incubation. Succinic acid monomethyl esters, pyruvic acid, L-alanine, L-glutamic acid, L-asparagine , α hydroxy butyric acid, β Hydroxy butyric acid , α ketoglutaric acid were utilizedon prolonged incubation. Results of Biolog® plates of *Enterobacter* sp. (S_4), *Bacillus licheniformis* (DI_2) and *Bacillus cereus* (GP_1) are shown in Photograph 9, 10 and 11.



Photograph 9. Biolog® plate of *Enterobacter* sp.



Photograph 10. Biolog® plate of *Bacillus cereus*

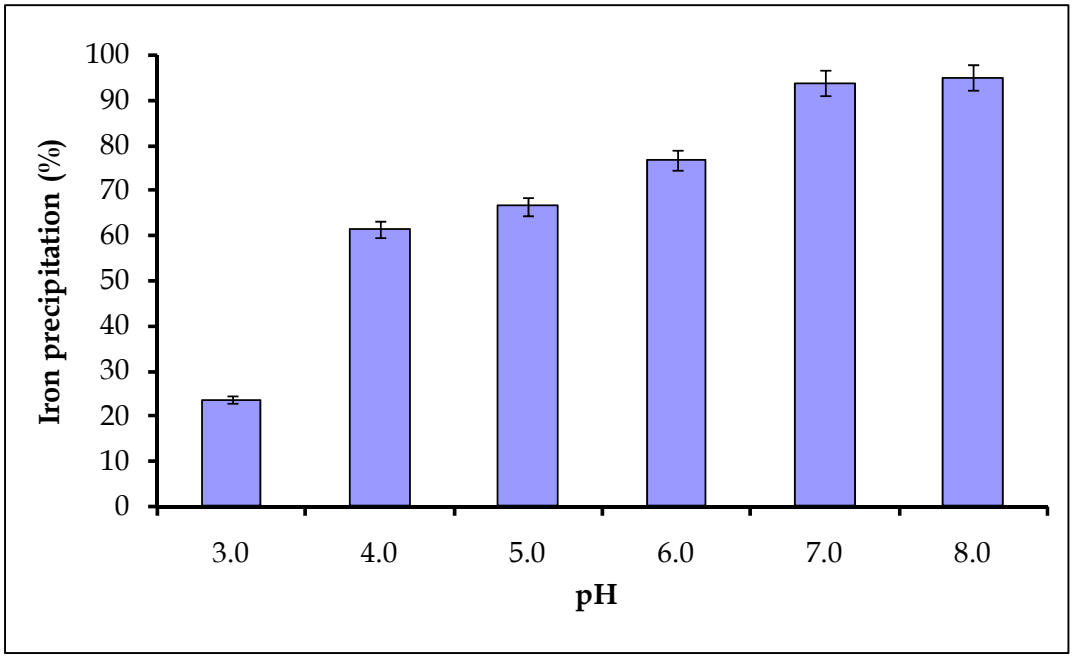


Photograph 11. Biolog® plate of *Bacillus licheniformis*

Influence of pH on iron bioprecipitation

The pH profile of *Enterobacter* was studied from 3.0 to 8.0. As shown in Graph 17, with the increase in pH, percentage of iron precipitation also increased.

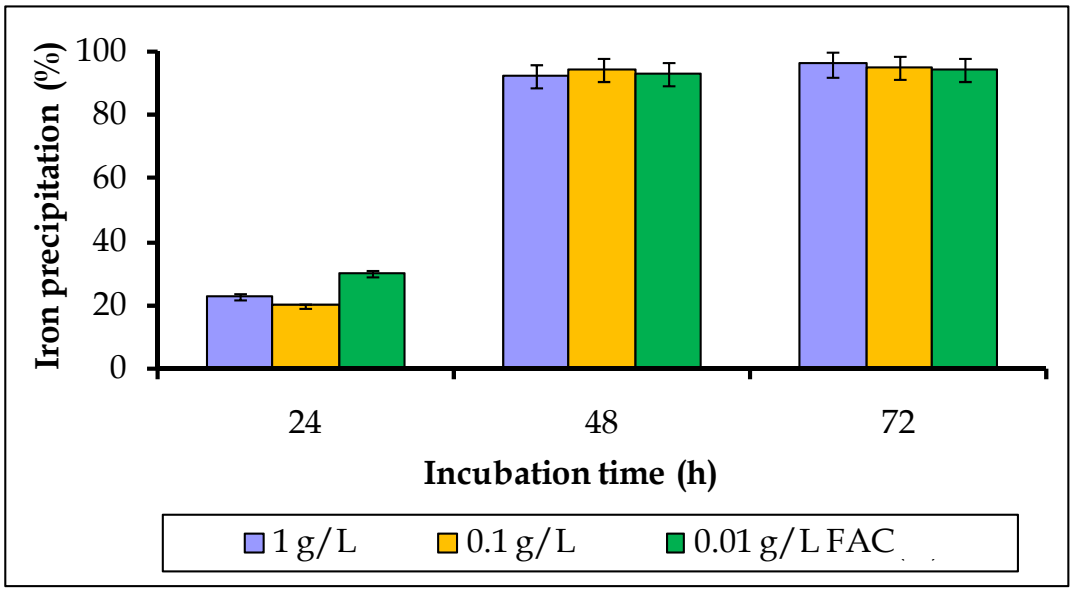
Graph 17. Influence of pH on iron precipitation by *Enterobacter* sp. (S_4)



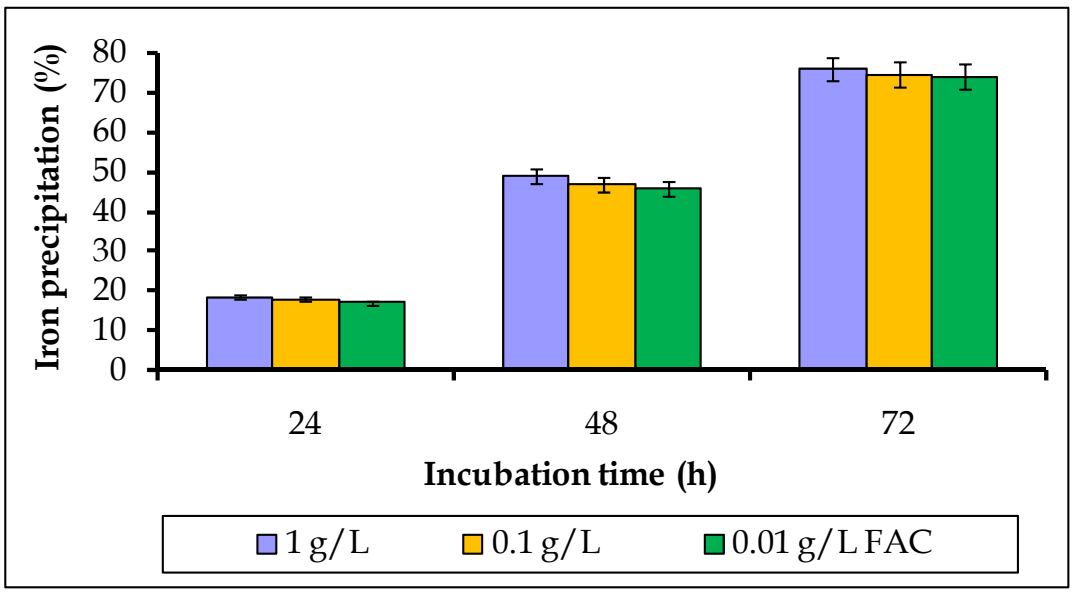
Influence of ferric ammonium citrate concentration on iron precipitation

Enterobacter sp. showed significant iron precipitating capacity that remove 96, 95 and 94% iron at 1.0, 0.1 and 0.01 g/L ferric ammonium citrate) in citrate broth medium. *Bacillus cereus* and *Bacillus licheniformis* removed only 76, 74.5 and 74% and 70, 69.5 and 68% iron respectively, the data are shown in Graphs 18-20.

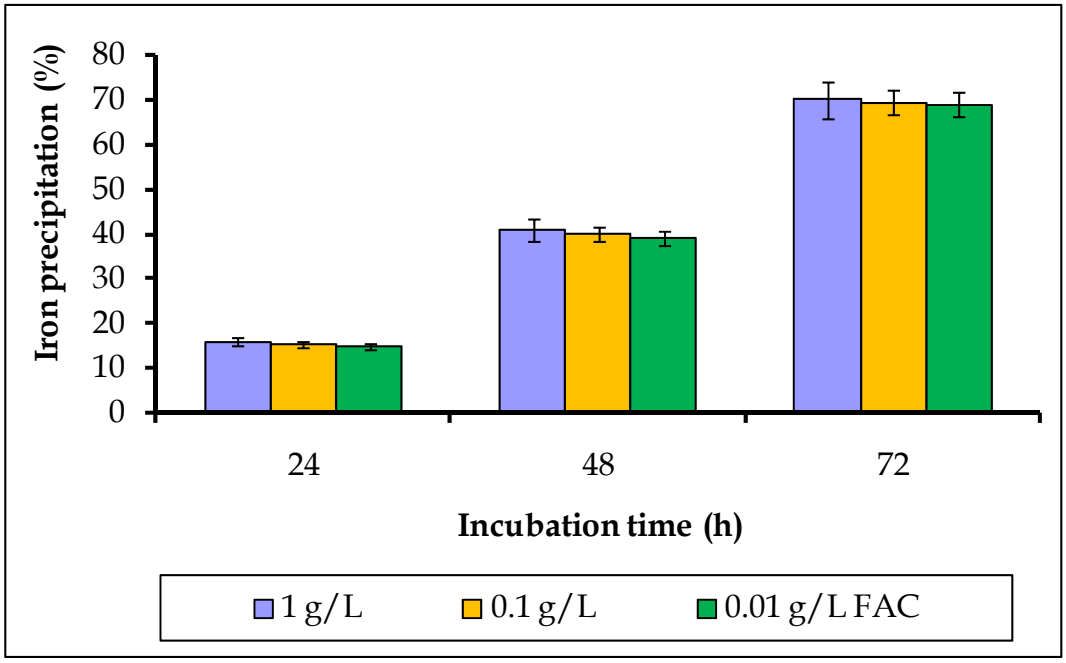
Graph 18. Comparison of iron precipitation by *Enterobacter* sp. (S_4) at various concentration of FAC



Graph 19. Comparison of iron precipitation of *Bacillus cereus* (GP_1) at various concentration of FAC



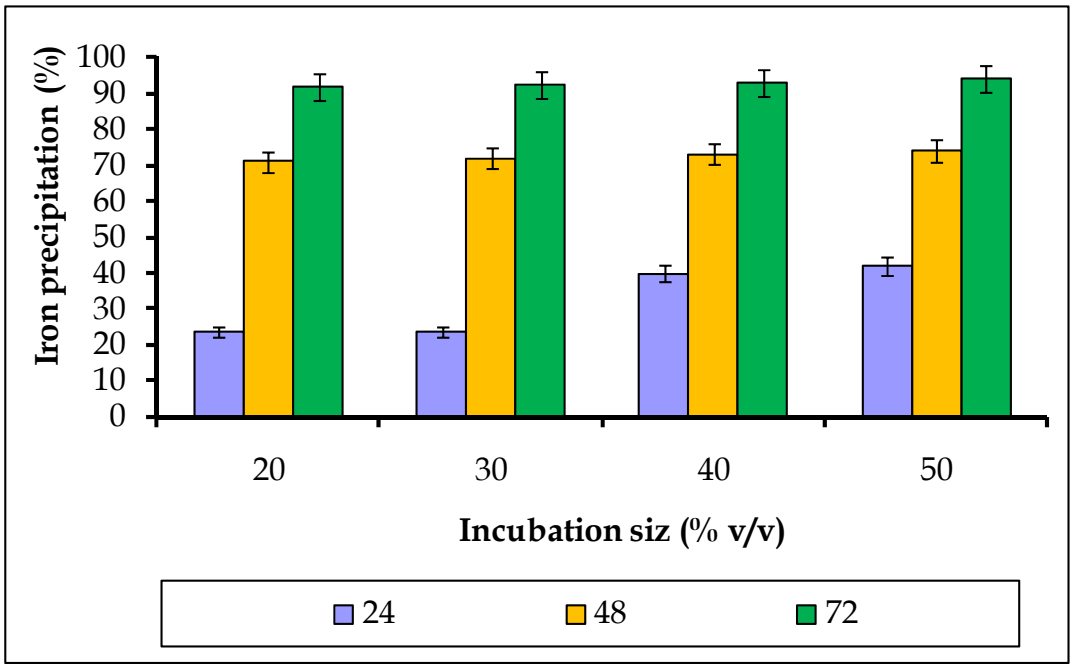
Graph 20. Comparison of iron precipitation of *Bacillus licheniformis* at various concentration of FAC



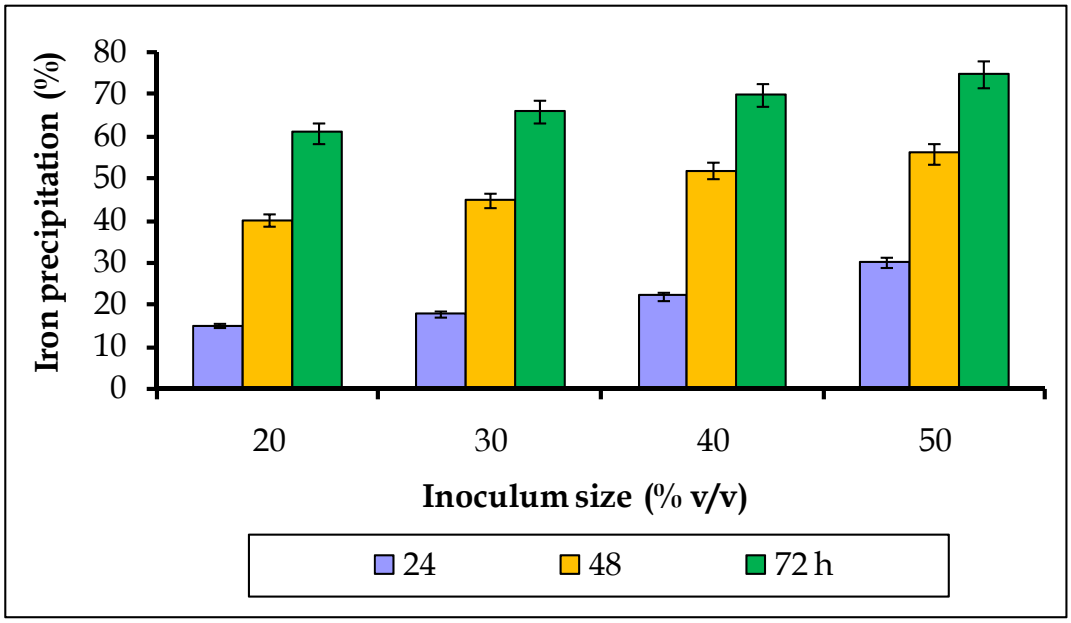
Influence of inoculum size on iron bioprecipitation

As can be seen from Graphs 21-23, when inoculum size was increased to 20, 30, 40 and 50%v/v, iron precipitation was found to be 68.6, 73.98, 79.5 and 94% for *Enterobacter* sp., 61, 66, 70 and 75% for *Bacillus cereus*, where as for *Bacillus licheniformis* it was 52, 58, 62 and 69% respectively.

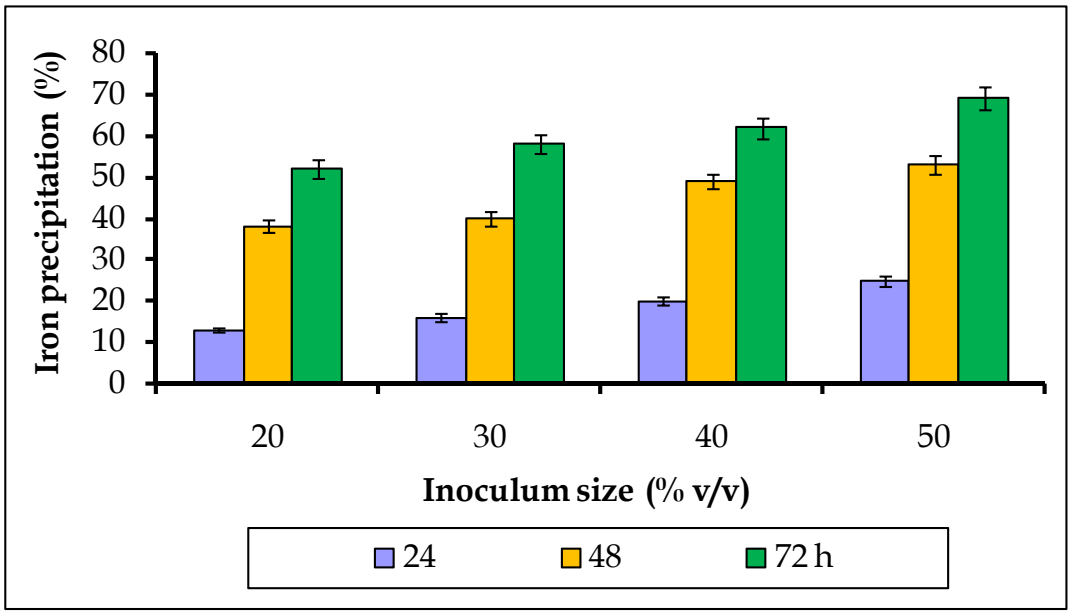
Graph 21. Iron precipitation by *Enterobacter* sp. (S_4) at different inoculum size



Graph 22. Iron precipitation by *Bacillus cereus* (GP_1) at different inoculum size



Graph 23. Iron precipitation by *Bacillus licheniformis* (DI_2) at different inoculum size



The main product of biological oxidation of iron is usually a mixture of poorly ordered iron oxides often containing significant amount of organic matter. The intermixing of iron oxides, organic material and bacterial biomass produces complex multiple sorbing solids, which exhibit unique metal retention properties. Arsenic can be removed by direct adsorption or co-precipitation on the preformed biogenic iron oxides. There is a report of As^{3+} removal by iron precipitating bacteria (Katsoyiannis, 2004). A study of iron removal between 30-60 °C and pH 4-9 by pure *Aeromonas* sp. and mixed culture of iron resistant microbes showed maximum removal of 45% (pH 8) and 90% (pH 9) respectively in 60-72 h by using synthetic ferric citrate medium containing 650 mg/L Fe^{3+} with ammonium chloride as nitrogen source was reported by Gopalan, 1993.

Chapter 4

Copper Bioremoval

Introduction

Properties

Copper, which is one of the earliest known metals occurs naturally in rock, soil, water and also in plants and animals (Tansupo et al, 2008). Copper is generated as a pollutant from mining process as well as in effluents from various industries, including tanning, metal processing, electroplating, automobile and pharmaceutical industry (Shah, et. al 1999; Qureshi et. al 2001). Copper is a reddish brown element in the transition metals family of periodic table. It has two oxidation states Cu^{1+} (the cuprous) and Cu^{2+} (the cupric). The compounds of first states are less stable, tending to be oxidized to Cu^{2+} even by oxygen of the air. (http://www.helium.com/items).

Occurrence

Copper is malleable, ductile and extremely good conductor of both heat and electricity. Today copper mined as major deposits in Indonesia, Chile, USA, Canada and Australia (http://www.lenntech.com/periodic/elements/cu.htm). Pure copper occurs rarely in nature. Usually copper found in the form of minerals such as azurite, malachite, bornite, chalcopyrite, covelite and chalcocite (http://enviornmentalchemistry.com/yogi). Copper has 29 isotopes ranging in atomic mass from 52 to 80. Twenty seven isotopes are radioactive and do not occur in nature (http://en.wikipedia.org). Some of the properties of copper are shown in Table 20 and image of copper is shown in Figure 9.

Table 20. Properties of copper

Property	Value
Atomic number	29
Atomic mass (g/mol)	63.546
Electro negativity according to Pauling	1.9
Density (g/cm^3 at 20 ºC)	8.9
Melting point (ºC)	1083
Boiling point (ºC)	2595
Vanderwal radius (nm)	0.128
Ionic radius (nm)	0.096 (+1); 0.069 (+3)
Isotopes	6
Energy of first ionization kJ/mol	743.5
Energy of second ionization kJ/mol	1946
Standard potential (Cu^+/Cu)	+0.522
(Cu^{2+}/Cu)	+0.345

Data adapted and modified from (http://www.lenntech.com/periodic)

Figure 9. Image of copper mineral.

(http://oecotextiles.files.wordpress.com /2010/11/copper.jpg)

Functions of copper

Copper can be found in several food, drinking water and in air, because of that we absorb significant quantities of copper each day by eating, drinking and breathing (http://www.lenntech.com /periodic/elements/cu.htm). Copper is an essential trace mineral, which is important for both physical and mental health (http://www.drlwilson.com). Copper is well distributed in the body. It occurs in liver, muscles and bones. Copper is transported in the bloodstream on a plasma protein called ceruloplasmin (http:// en.wikipedia.org/wiki/copper). Copper is required for estrogen metabolism and is also needed in the final steps of the Kreb's cycle called the electron transport system (http://www.drlwilson.com). Copper is involved in iron incorporation into haemoglobin (http://www.diagnose-me.com). Copper maintains the normal functioning of the brain and nervous system as it is required for the synthesis and metabolism of neurotransmitter. Copper act as antioxidant and protects against free radical damage. It is also involved with vitamin C to make elastin, an important constituent of the connective tissue and helps in bone formation (http://www.copperwiki.org). Copper is used in day to day activities as well as in many industrial processes. Some of its applications are given below.

Applications

1. The semiconductor industry is changing from aluminium to copper interconnection for memory application. This is primarily driven by the need for higher performance by speed of device (http://files.shareholder.com/downloads/NVLS/).

2. Copper is ideal for electrical wiring because it easily works, can be drawn into fine wire and has a high electrical conductivity (http://www.lenntech.com/periodic/elements/cu.htm).
3. It is useful for electrical work in electronic components, coins, valves and part of pumps and engine, electrical machine, electromagnetic motors, generators and transformers, electrical switching and vacuum tubes.
4. Good thermal conduction by copper makes it useful for heat sinks and in heat exchanger. Copper is supplied for industrial and commercial use in fine grained polycrystalline forms. It has excellent bronzing and soldering properties.
5. It is used extensively in refrigeration and air conditioning equipment because of its ease of fabrication and soldering.
6. It is used in copper plumbing fitting and compression tube, doorknobs, roofing and copper water heating cylinder.
7. Copper 64 can be used as a positron emission tomography radio trace for medical imaging.
8. Copper sulphate is used as fungicide and algal control in domestic lakes and pond.

Copper toxicity

Copper toxicity is based on the production of hyper oxide radicals and on interaction with cell membrane (Nies, 1999). The World Health Organization (WHO) recommended a maximum acceptable concentration of Cu^{2+} in drinking water 1.5 mg/L (Davis, 2010). Toxicity of copper occur from acidic food that has been cooked with copper cookware's and copper sulphate added to drinking water,

copper compound used in swimming pools. Acidic water such as rain water left standing in copper plumping pipes can be source of copper toxicity (http://en.wikipedia.org/wiki; http://www.diagnose-me.com/copper). Excessive amount of Cu^{2+} can cause serious health issues (Dannis, 2010). Aquatic organisms, especially certain species of crustaceans and fishes, have the highest sensitivity to copper toxicity (http://icpvegetation.ceh.ac.uk/Reports/).

Symptoms

1. Fatigue
2. Anorexia (lack of appetite)
3. Depression
4. Anxiety
5. Migraine
6. Premenstrual syndromes (http://www.arltma.com/CopperTox)
7. Respiratory difficulty and gastrointestinal bleeding (Danis, 2010)
8. Liver damage, coma and death (http://www.copperwiki. org)
9. Vomiting, diarrhea, stomach-ache and dizziness (http://www.lenntech.com/periodic/elements/cu.htm).
10. Wilson's disease or hepatolenticular degeneration is an autosomal recessive genetic disorder in which copper accumulates in tissues. It is treated with medication that reduces copper absorption or removes the excess copper from the body (http://en.wikipedia.org/wiki/Wilson's_disease).

Conventional methods for removing dissolved heavy metal ions from waste water include chemical precipitation, chemical oxidation and reduction, ion exchange and filtrations. Disadvantages of these

techniques are incomplete metal removal, the need for expensive equipment and monitoring system, energy requirement and generation of toxic sludge or other waste products that require disposal (Shetty, 2009).

Bioremoval of copper

Many authors have reported copper remediation by bacteria, fungi, yeast and algae (Cervantes and Guitierrez, 1994; Qureshi et. al, 2001; Beolchini et. al, 2004; Vijayraghvan et. al 2004; Zaki and Farang, 2010). Both live and dead biomass can be used for copper removal. Spent dead biomass was found to sequester copper ions from aqueous solution. Copper sorption from aqueous solution by *Streptomyces* was reported by Shah et. al in 1999. Bioremediation, using bacteria, fungi or plants is often regarded as a relatively inexpensive and efficient way of cleaning up wastes, sediments or soil contaminated with heavy metals (Huang et.al, 2005). An important aspect of biosorption is that it can be carried out by both metabolically active and inactive cells (Zaki and Farang, 2010). Bacterial mechanism of copper resistance are related to reduced copper transport, enhanced efflux of cupric ions, or copper complexation by cell components. Copper tolerance in fungi has also been reported by diverse mechanisms involving trapping of the metal by cell wall components, extra cellular chelation or precipitation by secreted metabolites (Cervantes and Guitirrez, 1994) Both live and dead biomass can be used to remove metals but maintaining a viable biomass during metal adsorption is difficult because it requires continuous supply of nutrients and toxicity of

metal for microorganisms might take place (Beolchini et. al, 2004). Adsorption capacity of copper by *Bacillus subtilis, Enterobacter aerogenes, Acidithiobacillus ferrooxidans, Brevibacterium* sp. were also reported in literature (Huang et.al, 2005). The maximum copper uptake by *Sphaerotilus natan* was about 0.7 mM/g of biomass (Beolchini et. al, 2004). *Pseudomonas putida* CZ1 was capable of removing about 87.2% copper with specific biosorption capacity of 24.2 mg/L biomass because of its high metal uptake capacity in aerobic condition. This bacterium may be potentially applicable in bioreactor or *in-situ* bioremediation of heavy metal contaminated aqueous or soil system (Xincai et. al, 2006). Sulphate reducing bacteria *Desulfovibrio* sp. isolated from submerged soil samples of paddy fields effectively precipitated copper from aqueous solution with maximum removal of 75% at 25 ppm Cu^{2+} (Panchanadikar and Kar, 1993).

Adsorption of Cu^{2+} ions by green dried algae *Cladophora* sp. was studied in packed bed column reactor (Aksu and Kutsal, 1998). The *sargassum* sp. brown seaweed was used as a biosorbent for copper removal (Das, 2008). *Ulva reticulate*, marine green algae are used for copper removal from aqueous solution (Vijayaraghvan et. al, 2004).

Iron oxides, hydroxides and oxide hydroxides consist of arrays of Fe ions and hydroxide ions. In comparison with minerals existing in soil, iron oxides have relatively high surface area and surface charge and they often regulate free metal concentration in soil through adsorption reactions. Many researchers have applied the iron oxide to the treatment of heavy metals from metal bearing tap or

wastewater. Iron oxide coated Low Expanded Clay Aggregates (Fe-LECA) was used as a new adsorbent to remove copper ions from water (Alli, 2004). In this context, in our study iron bioprecipitation was investigated for its role in copper removal from aqueous solution.

Materials and Methods

Screening of isolates

Thirty isolates were obtained on citrate agar medium, from different water and soil samples as shown in Table 10 of (Chapter 1). Out of them, three isolates were screened for copper bioremoval.

Copper bioremoval parameters

Shake flask study of copper removal

Experiments were conducted in 250 ml Erlenmeyer flasks with total system of 50 ml of optimized citrate broth (Appendix I) containing 10, 20, 40, and 80 ppm of copper. Actively growing 10% (v/v) culture having 4.1×10^8 cells/ml of *Enterobacter* sp. (S_4) or *Bacillus cereus* (GP_1) or *Bacillus licheniformis* (DI_2) were used as inoculum. Negative control for each was kept devoid of culture. Flasks were incubated in environmental orbital shaker (Newtronics, India) rotating at 150 rpm at 32±2ºC. At regular time interval 5 ml broth was taken and centrifuged at 9000 g for 15 min (Remi C24, India). The biomass was separated and desired amount of supernatant was taken for copper analysis. The percentage of copper removal was calculated based on the amount of initial copper present in the system.

Optimization of pH

To check the pH optima for copper removal, all the flasks with 50 ml citrate broth medium containing 10% v/v actively growing inoculum of *Enterobacter* sp. having 4.1×10^8 cells/ml were adjusted at pH 3.0, 5.0 and 7.0 by 0.1N HCl. In each flask 20 ppm of copper was added

and was kept in environmental orbital shaker at 150 rpm at 32±2ºC. Remaining copper was estimated by standard diethyl dithiocarbamate complex method (Vogel, 1961) (Appendix II).

Copper removal with and without ferric ammonium citrate

Experiments were conducted in 250 ml Erlenmeyer flasks with total system of 50 ml of medium with and without ferric ammonium citrate containing 20 ppm of copper. Actively growing 10% v/v culture having 4×10^8 cells/ml of *Enterobacter* sp. was used as inoculum. Flasks were incubated in environmental orbital shaker (Newtronics, India) rotating at 150 rpm. The sample was centrifuged at 9000g for 15 min (Remi C24, India). Supernatant was collected periodically for copper analysis.

Column study

The biofilm reactors have been used for several processes and gained acceptance for its efficiency (Shah, 2005). The most applied method for metal removal is use of cells immobilized as biofilm on inert supports. Ideal support should have large surface area, but should be porous enough to enable high flow rates and minimal clogging. The biomass could be immobilized directly or it may be modified by chemical or physical treatment to improve its biosorption efficiency. Microbial cells can be immobilized by using variety of support materials including agar, cellulose, alginate, polyacrylamide toluene disocyanate and gluteraldehyde (Modi, 1996). In addition to this, materials like glass, metal sheets, plastic, wood shaving, sand, crushed rocks can also be used for immobilizing live cells. These systems have been used in variety of reactors including rotating

biological contractor, fixed bed reactor, trickle filter fluidized bed reactor and airlift bioreactor (Modi, 1996).

A glass column of 38 cm length, 2.74 cm of inner diameter and of 3.6 cm of outer diameter was used in the study. Glass wool was inserted at the lower end of the column and the column was filled up to 10 cm height with polystyrene beads. The average weight, length and width of polystyrene bead were 0.0246 g, 4.12 mm and 2.99 mm respectively. The surface area and volume of bead was 133.5 mm^2 and 115.6 mm^3 respectively. The total volume of column was 130 ml with working volume of 50 ml. The experiment was performed both with aeration and without aeration. Aeration was provided to the column by the aerator. For the development of biofilm actively growing culture of *Enterobacter* sp. in citrate broth medium was passed through the column for nine days. The entire medium was drained gradually. During study the copper concentration was increased from 20 to 200 ppm in the medium and was passed through the column and allowed to react for different time period and the copper was estimated from the effluent of each cycle spectrophotometrically as described in previous part. Picture of column reactor used for copper bioremoval study is shown in Photograph 12 (A) and (B).

A **B**

Photograph 12. Laboratory scale column reactor for copper bioremoval (A) citrate broth with *Enterobacter* sp. (B) polystyrene beads after biofilm formation.

Results and Discussions

Screening of isolates

Copper removal by three isolates is shown in Table 21. Isolate S_4 showed 80% of copper removal, while isolates GP_1 and DI_2 removed 76% and 71% copper respectively from the medium. Among the three isolates, isolates S_4 gave better result as compared to isolate GP_1 and DI_2, hence it was selected for further study.

Table 21. Screening of isolates for copper bioremoval

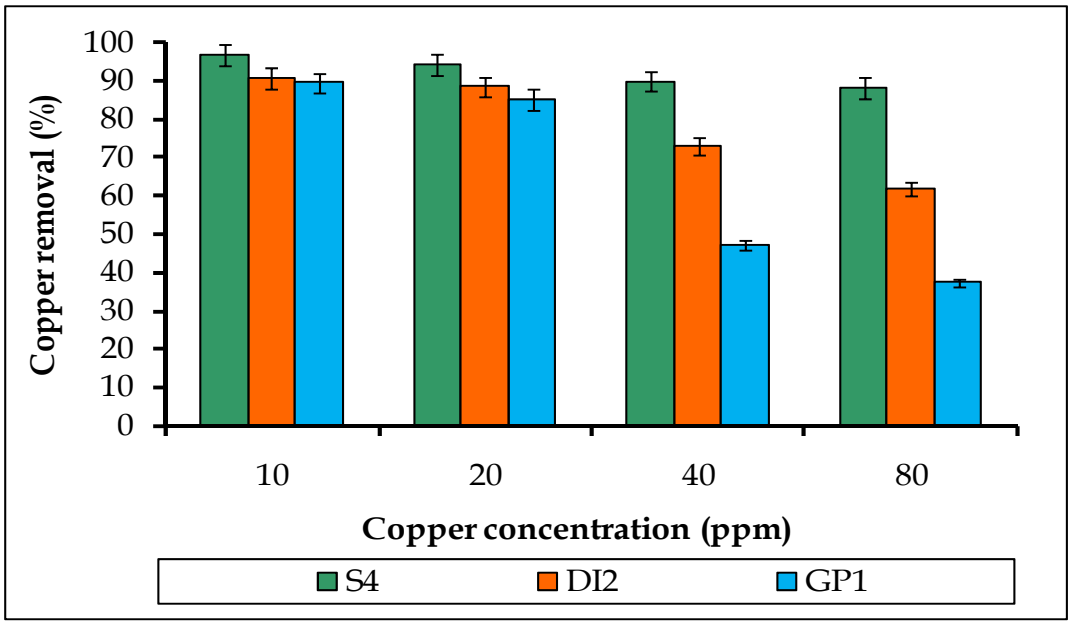
Isolates	Copper bioremoval (%)
S_4	80
DI_2	76
GP_1	71

Shake flask study of copper bioremoval

Copper bioremoval in shake flask study by the three iron precipitating organisms is shown in Graph 24. Among the studied three isolates, *Enterobacter* sp. (S_4) showed maximum copper removal irrespective of the copper concentration studied, where as *Bacillus cereus* (GP_1) showed the lowest copper removal. When the initial copper concentration was increased from 10 to 80 ppm *Bacillus cereus* (GP_1) copper removal efficiency decreased by 52% on the other hand, only 29 and 8% decrease in copper removal was observed with *Bacillus licheniformis* (DI_2) and *Enterobacter* sp. (S_4) respectively. This result indicates that *Enterobacter* sp. is the organism of choice for

copper removal. Thus all further experiments were done with *Enterobacter* sp. (S_4)

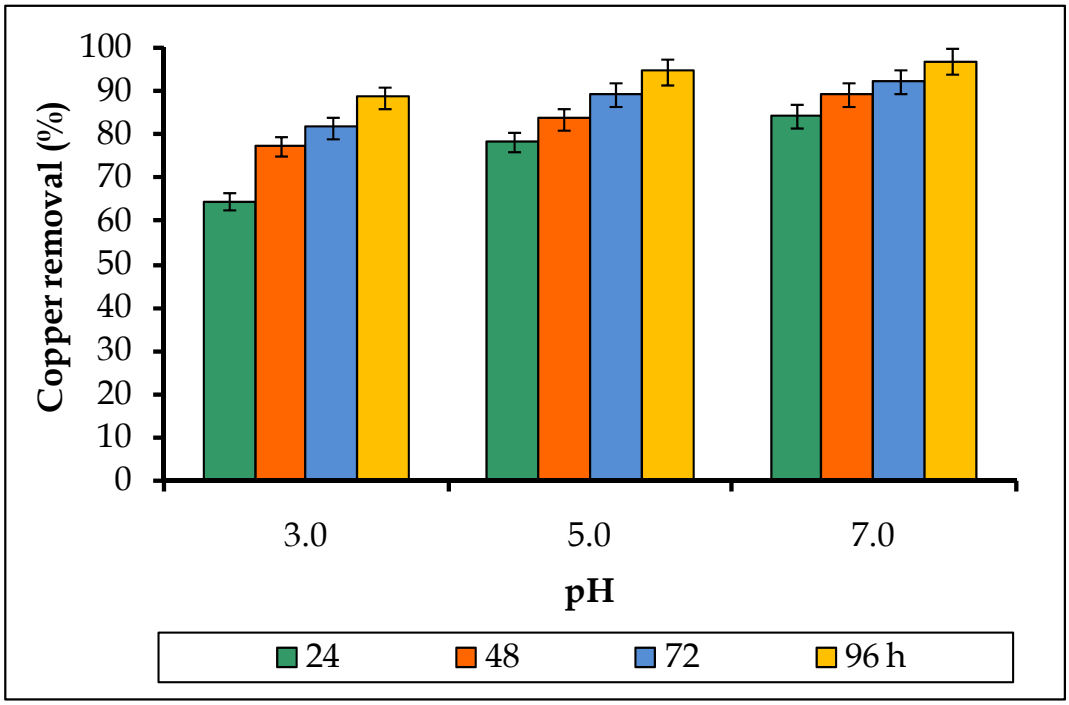
Graph 24. Shake flask study of copper bioremoval by various isolates



Optimization of pH

The pH condition is of prime importance in determining the mobility of metal (Tansupo et. al, 2008). Both iron and copper precipitation is greatly influenced by the environmental pH. Thus, influence of pH on copper bioremoval by *Enterobacter* sp. (S_4) was studied and results are shown in Graph 25. In 24 h of incubation 84.2% copper was removed from a medium containing 20 ppm copper at pH 7.0 However, only 78.3% and 64.5% copper removal was observed at pH 5.0 and pH 3.0 respectively.

Graph 25. Effect of pH on copper bioremoval by *Enterobacter* sp. (S_4)

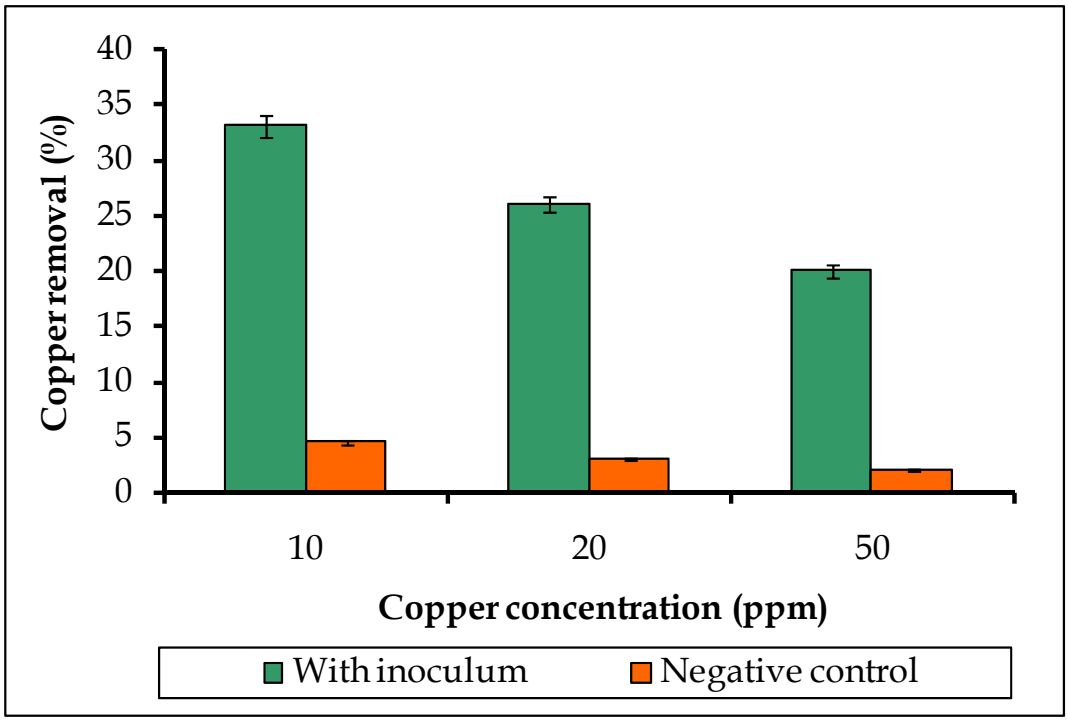


The trend continues even for prolonged incubation of 96 h. However, the difference in the copper removal between pH 3.0 and pH 7.0 was narrowed down at 96 h as compared to the result obtained at 24 h of incubation. This is obviously due to very less amount of copper remaining as the incubation time increases in case of the experiment done at pH 7.0. The obtained result suggests the possibility of use of the organisms in wide range of pH for copper removal.

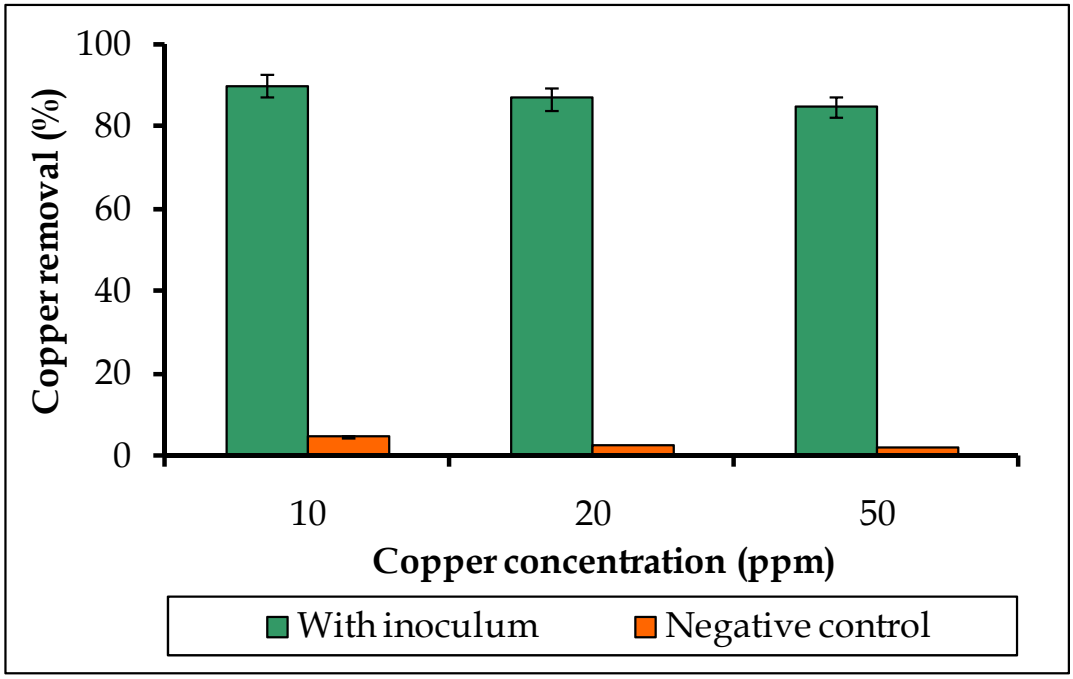
Influence of ferric ammonium citrate on copper removal was studied and the results are shown in Graph 26 and 27. The presence of 1 g/L of ferric ammonium citrate showed 2.7 to 4.24 fold more copper removal as compared to the test in absence of ferric ammonium citrate. The beneficial effect of ferric ammonium citrate was becoming

more and more prominent as the concentration of copper was increased from 10 to 50 ppm. This indicated that the biological activity with ferric ammonium citrate play crucial role in copper remediation. As in case of presence and absence of ferric ammonium citrate in the medium without inoculation of *Enterobacter* sp. showed as less as 2 to 5% copper removal even at the end of 72 h of incubation.

Graph 26. Copper bioremoval by *Enterobacter* sp. (S_4) without FAC



Graph 27. Copper bioremoval by *Enterobacter* sp. (S_4) with FAC



Influence of ferric ammonium citrate concentration in the media was studied for copper bioremoval in shake flask and column experiment and results are shown in Table 22. Column experiment showed better removal as compared to shake flask study. This is obviously due to larger biomass present in the column as a biofilm. Even, when 0.01 g/L ferric ammonium citrate was present, 72.2 and 87.5% copper was removed from 50 ppm of copper containing medium in shake flask and column study respectively. However, 0.1g/L of ferric ammonium citrate gave optimal result in removing 50 ppm copper in flask as well as column.

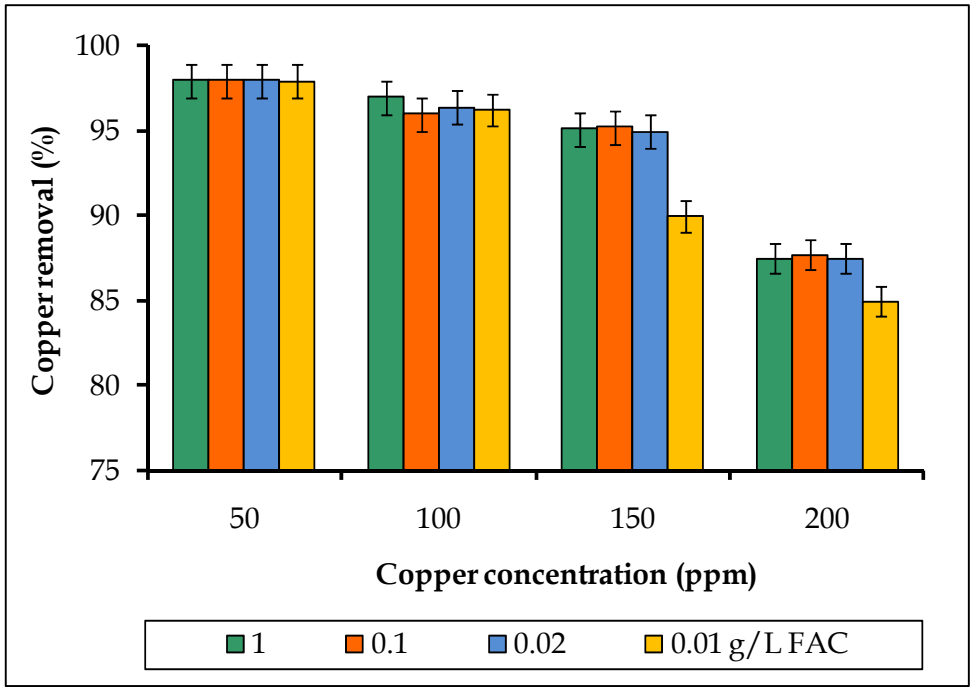
Table 22. Comparison of shake flask and column study for copper bioremoval

Ferric ammonium citrate (g/L)	1	0.1	0.02	0.01
50 ppm (Flask)	85	82	78.5	72.2
50 ppm (Column)	98	97.36	95.12	87.5

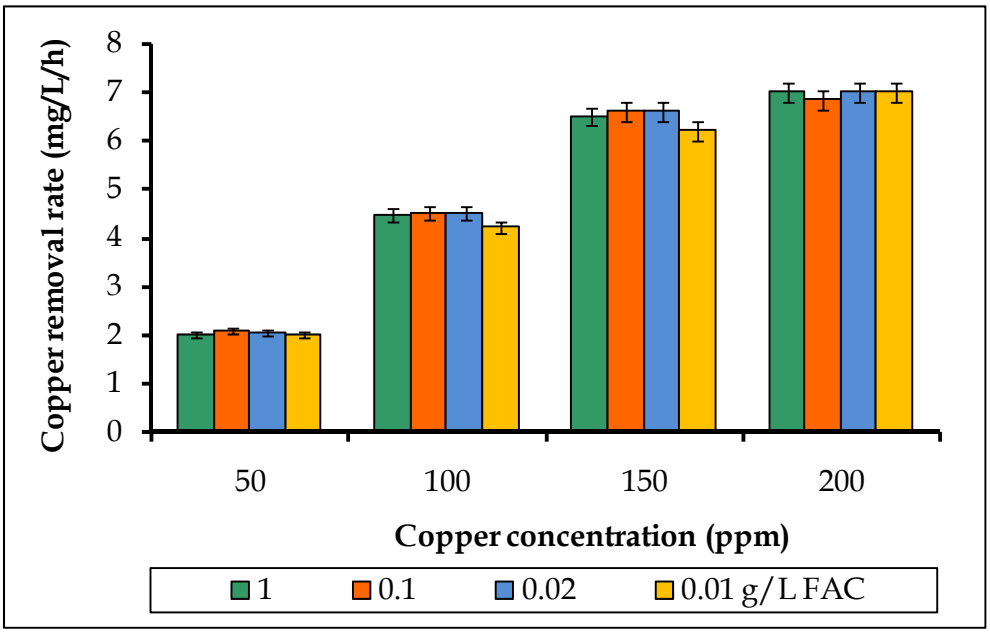
Column study

Column study showed better result as compared to shake flask experiment. Thus, influence of ferric ammonium citrate concentration for removal of different copper concentrations was studied in column and results are shown in Graph 28 (A and B). Under the experimental condition, the range of ferric ammonium citrate studied showed less than 15% difference in copper removal, when ferric ammonium citrate concentration was decreased from 1 g/L to as low as 0.01 g/L irrespective of the amount of copper present in the solution i.e. 50-200 ppm. This finding showed that more than 80% copper was removed even when as low as 0.01 g/L of ferric ammonium citrate was present in the medium. When the results are interpreted in terms of copper removal rate, there was substantial influence of amount of copper present. The removal rate was directly proportional to the amount of copper concentration in the system. Under the condition studied, the copper removal rate varied between 2 to 7 mg/L/h for 50 and 200 ppm of copper concentration respectively in aerobic condition.

Graph 28 (A). Effect of FAC on copper bioremoval percentage with aeration by *Enterobacter* sp. (S_4) in column

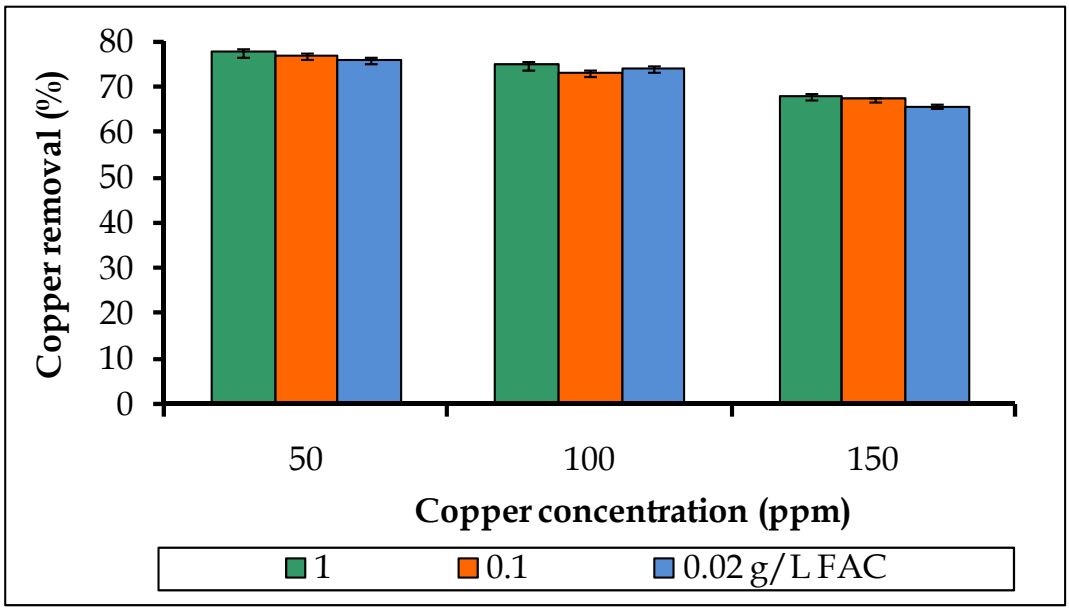


Graph 28 (B). Effect of FAC on copper bioremoval rate with aeration by *Enterobacter* sp. (S_4) in column

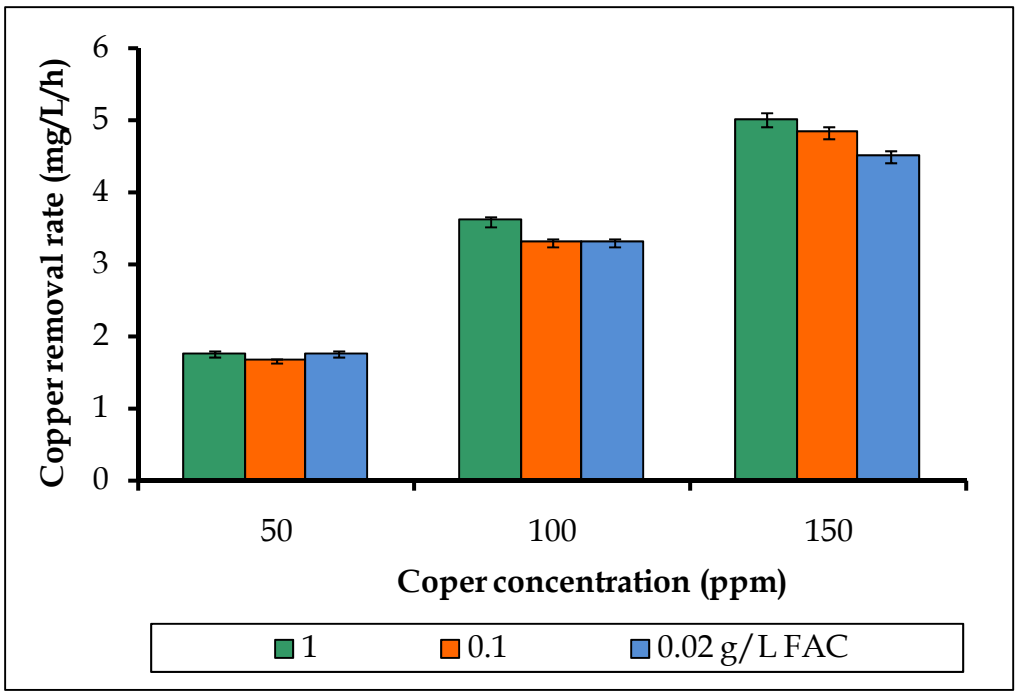


The result of ferric ammonium citrate concentration in the absence of aeration was also studied and the results are shown in Graph 29 (A and B).The highest removal was 77.8% for 50 ppm of copper and 68.16% for 100 ppm of copper, which was 20 and 30% less as compared to copper removal in the presence of aeration (Graph 28 A and B) for 50 and 100 ppm copper respectively. Similarly, decrease in copper removal rate was also noticed in absence of aeration as it can be seen from Graph 29 (A and B). This could be due to the influence of aeration, which enhanced growth as well as iron precipitation that could be responsible for higher copper removal and enhanced rate of removal.

Graph 29 (A). Effect of FAC concentration on percent copper bioremoval without aeration by *Enterobacter* sp. (S_4) in column



Graph 29 (B). Influence of FAC concentration on rate of copper bioremoval without aeration by *Enterobacter* sp. (S_4) in column



Chapter 5

Mercury Bioremoval

Introduction

Properties

Mercury is one of the most toxic pollutants, threatening our health and ecosystem (Mathivanan et al., 2010). Mercury is a heavy silvery white liquid metal. It alloys easily with many metals. These alloys are called amalgams (http://www.lenntech.com/periodic/elements/hg .com). It forms salts in two ionic states Hg^{+} and Hg^{2+}. Hg^{2+} salts are more common than Hg^{+} salts (Environmental Health Criteria 86, 1989). Properties and image of mercury are shown in Table 23 and Figure 10.

Table 23. Properties of mercury

Property	Value
Atomic number	80
Atomic mass (g/mol)	200.59
Electro negativity according to Pauling	1.9
Density (g/cm^3 at 20ºC)	13.6
Melting point (ºC)	38.9
Boiling point (ºC)	356.6
Vanderwaal's radius (nm)	0.157
Ionic radius (nm)	0.11 nm (+2)
Isotopes	12
Electronic shell	(Xe) 4f14 5d10 6s2
Energy of first ionization (kJ/mol)	1004.6
Energy of second ionization (kJ/mol)	1796
Energy of third ionization (kJ/mol)	3294
Standard potential	+0.854V (Hg^{2+}/Hg)

Data adapted and modified from (http://www.lenntech.com/periodic/elements /hg.htm).

Figure 10. Image of mercury (http://3.bp.blogspot.com/_HrsMw-at3Rs/TPfoaPpY0dI/AAAAAAAAABU/mGQzDGIJRQE/s1600/mercury2.jpg

Occurrence

It is commonly found as sulphide ore such as cinnabar in Spain, Russia, Italy and Slovenia (http://www.lenntech.com/periodic/elements/hg.com). The annual global emission of mercury ranged between 4800-8300 tons per year. Mercury is released into the hydrosphere, atmosphere and biosphere as a consequence of natural and anthropogenic processes. It is cycled in the environment and undergoes transformations of its chemical forms. In atmosphere, mercury moves in its volatile forms such as elemental vapour or methyl mercury as well as particulate bound forms (Morita et. al, 1998). Mercury cycling in aquatic environment is shown in Figure 11.

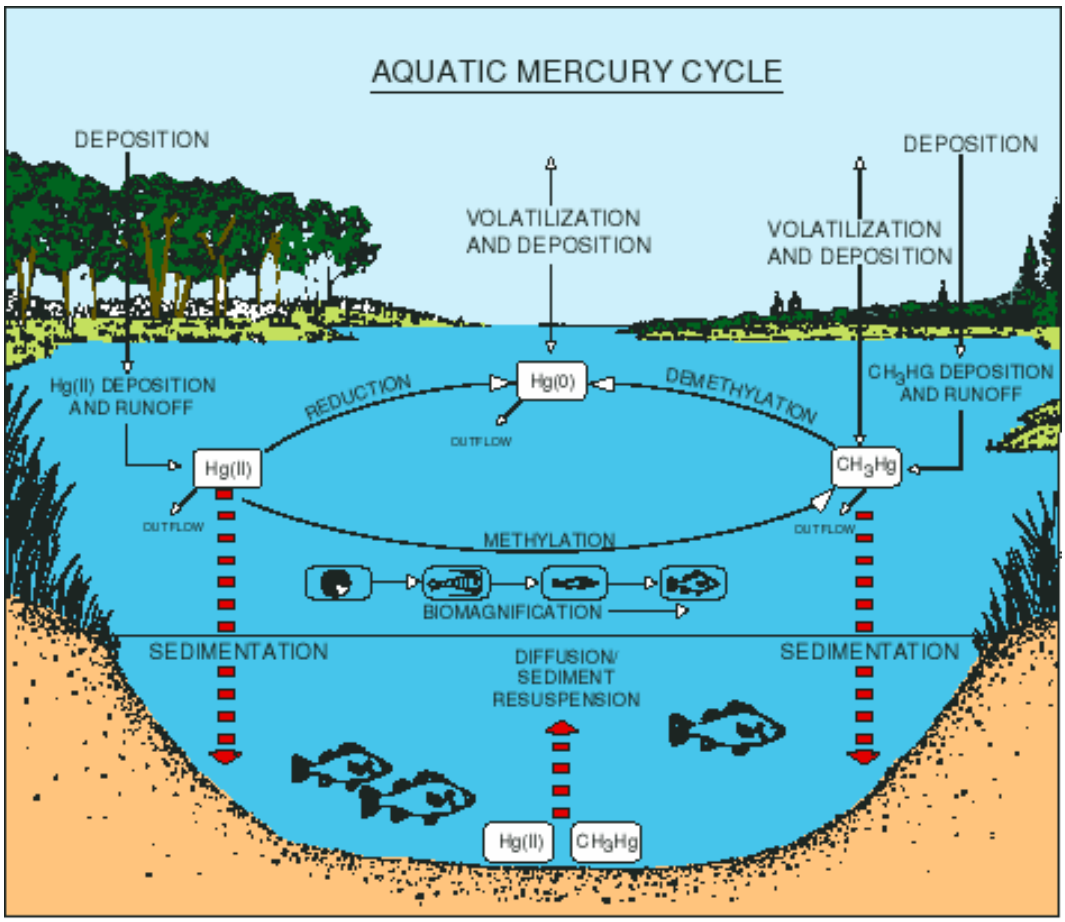


Figure 11 . Mercury cycling pathways in aquatic environment (http://wi.water.usgs.gov/mercury/images/mercury-cycle.gif)

Source in the environment

Environmental contamination of mercury is caused by several industries, petrochemical products, mining activity, painting materials as well as by agricultural sources such as fertilizers and fungicidal spray (Rezaee et. al, 2005). Natural release of mercury into the environment refers to the mobilization and release of geologically bound mercury by natural biotic and abiotic processes with mass transfer of mercury into the atmosphere. Volcanic eruption and geothermal activities contribute significantly to the natural emissions

(Okoronkwo et. al, 2007). The major source of human exposure to mercury is consumption of seafood (De, 2004).

Toxicity of chemical species of mercury

Mercury accumulates through food chain in ecosystem. Organic and inorganic forms are not equally toxic to biota (Santra, 2005). Mercury contamination in soils and sediments is harmful if converted to methylmercury, the more toxic and bioaccumulative form of the metal. Methylation of mercury in the environment occurs primarily by anaerobic microorganisms such as sulphate reducing bacteria. Methyl mercury production rates generally depends upon the growth of these anaerobic organisms and also on the amount of inorganic mercury that is available for uptake into these bacteria (http://www.goldschmidt2010.org/abstracts/). The most toxic species are the organomercurials particularly methyl mercury which is soluble in fat, lipid section of membranes and brain tissue. Methyl mercury poisoning also leads to segregation of chromosomes, chromosome breakage in cells and inhibited cell division. Attachment of mercury to cell membrane inhibits active transport of sugars across the membrane and allows the passage of potassium to the membrane (Santra, 2005). Inorganic mercury forms are less harmful as compared to organic forms, because they bind strongly to soil component that reduces their availability and absorption. Organomercurials are highly toxic because of their movement across cell membranes and accumulation in membrane bound organells, inhibiting essential oxidative and photosynthetic pathways (Mathivanan et. al, 2010). The hazard arising from elemental mercury is owed to its vapour

pressure allowing it to be easily inhaled. Absorbed by the lungs, it enters the blood and circulated throughout the body including brain. Repeated or prolonged exposure mainly results in vasomotor disturbances, tremors and behavioural disturbances (De, 2004). The toxicity of mercury depends on its chemical species as shown in Table 24.

Table 24. Chemical and biochemical properties of mercury species (Santra, 2005)

Species	Chemical and biochemical properties
Hg^0	Elemental mercury: relatively inert and non toxic; vapour highly toxic when inhaled
Hg_2^{2+}	Mercurous ion: insoluble as chloride; low toxicity
Hg^{2+}	Mercuric ion: toxic but not easily transported across biological membranes.
RHg^+	Organomercurials: highly toxic, particularly methyl mercury, causes irreversible nerve and brain damage; easily transported across biological membranes; stored in fat tissue
R_2Hg	Diorganomercurials: low toxicity but can be converted to RHg^+ in acidic medium
HgS	Mercuric sulphide: highly insoluble and non toxic; trapped in soil in this form

Applications

Mercury finds variety of applications. Some of which are enlisted below.

1. Mercury is used in the production of electrical apparatus such as mercury vapour lamp, electrical switches, fluorescent tubes and batteries etc.
2. In agriculture industry, organomercurials and used as fungicides for seed dressings.
3. Mercury is used as filling material for dental cavities as silver amalgam.
4. Organic mercurials such as metaphin and mercurochrome exert a weak bacteriostatic action and are used as local antiseptics. Mercuric oxide is used as skin ointment.
5. HgS (Vermillion) is used as high grade paint pigment.
6. Mercury has higher density. Hence, it is also used in thermometer and barometer (http://www.lentech.com/; Environmental Health Criteria 86, 1989; Santra, 2005).

Symptoms of mercury toxicity

The cytotoxicity of mercury is due to their ability to get solubilized into lipids, to bind with sulphydryl group of membranes and enzymes to inhibit macromolecular synthesis, transcription and translation (Modi, 1996).

1. Mercury accumulates in kidney tissue directly causing renal toxicity, including protein urea or nephritic syndrome. Higher

concentration of mercury also causes impairment of pulmonary function of kidney and chest pain (Mortuzavi et. al, 2005).

2. Disruption of nervous system, brain function, DNA and chromosomal damage, allergic reactions like skin rashes and headache (http://www.lenntech.com/periodic/elements/hg.htm).
3. Irritability, restlessness, insomnia, drowsiness and loss of memory (http://www.mercurypoisoned.com/).
4. Minamata disease is a neurological syndrome caused by severe mercury poisoning for the first time in the world at Minamata city, Japan (http://www.symptoms101.com/; http://aileen archive.or.jp/minamata_en/). The symptoms includes blurred vision, slurred speech and loss of muscle control and these symptoms were followed by violent trembling, paralysis and even death. Children were born with tragic deformities and permanent mental retardation because of Chisso chemical plant had been releasing residues containing mercury into Minamata Bay. Since elemental mercury is not water soluble, it would sink into the bottom sediments and remain inert. Bacteria and fishes living in the sediments were able to convert metallic mercury into soluble methyl mercury, which was absorbed from the water and concentrated in the tissues of aquatic organisms. Those who ate them had been poisoned with it.

5. Infantile acrodynia also known as "Calomel disease" or "Pink disease" is a type of mercury poisoning in children, characterized by pain and pink discolouration of hands and feet (http://en.wikipedia.org/wiki/Mercury_poisoning).

Wood (1984) discussed six protective mechanisms available to microorganisms and certain higher organisms that increase their resistance to metal ions in general, and specifically to mercury. These mechanisms are:

- Efflux pumps that remove the ion from the cell, a process which requires energy.
- Enzymatic reduction to the less toxic elemental form.
- Chelation by intracellular polymers (not firmly established for mercury).
- Binding of mercury to cell surfaces.
- Precipitation of insoluble inorganic complexes, usually sulphides and oxides, at the cell surface.
- Biomethylation with subsequent transport through the cell membrane by simple diffusion. It is this last mechanism, biomethylation, which renders the mercury more toxic to higher life-forms. (Environmental Health Criteria 86, 1989).

Non-viable biomass of estuarine *Bacillus* sp. was employed for adsorbing Hg^{2+} ions from aqueous solution. The optimum pH for biosorption varies from 4.5 to 6.0 (Ruiz, 2006). Dissimilatory iron reducing bacteria (DIRB) play important role in mercury methylation. Mercury methylation by a *Geobacter* strain is reported (Kerin et. al, 2006). Metabolically active cells, inactive cells and dead biomass can accumulate metals. Living systems are employed mostly on the consideration that:

1. Biosorbents in live cell systems being a renewable source does not require replenishment when it gets saturated with metals.
2. Products of organisms such as H_2S, metallothioeins, phytochelatins etc. can be used for metal immobilization.

The physiochemical technology available to treat the soluble mercury containing waste is precipitation using sodium sulphate, coagulation with ammonium sulphate or iron salts. A polystyrene type cation exchanger has ability to remove mercury from large volume of waste water from pesticide industry and other types of technologies are available for removing mercury from water and waste water includes reverse osmosis, ultrafiltration, magnetic filtration, activated carbon adsorption and chemical reduction. Though, the above methods are applied to treat the effluents; they have one or the other disadvantages because these processes require high operational costs, enormous chemicals, process are tedious and time consuming and lead to secondary pollution (Modi, 1996).

Bioremoval of metal ion from polluted water has the potential to achieve greater performance at low cost as compared to conventional technologies. The use of microorganisms to sequester, precipitate or alter the oxidation state of various heavy metals has been extensively studied. Processes by which microorganisms interact with toxic metals are biosorption, extracellular precipitation and uptake by purified biopolymers and other specific molecules derived from microbial cells (Mortazavis et. al, 2005). Recent literature shows that bioremediation strategies including biotransformation, biosorption and bioprecipitation of mercurials have been developed (Mathivanan

et.al, 2010). Chelation therapy for acute inorganic mercury poisoning can be done with DMSA 2,3 dimercapto-1 propanesulfonic acid and dimercaprol (http://en.wikipedia.org/wiki/Mercury_poisoning).

Mercury resistant bacteria contain membrane associated proteins that selectively bind to Hg^{2+} and carry it across the cellular envelop, allowing subsequent reduction catalyzed by an intracellular enzyme mercuric reductase (Chang and Hong, 1994). *Staphylococcus aureus* isolated from BHEL (Bharath Heavy Electrical Limited), Tiruchirappalli showed better growth in presence of high $HgCl_2$ concentration indicating mercury resistance capacity (Mathivanan et.al, 2010). Removal of mercuric chloride by *Pseudomonas putida* isolated from sludge of chloralkali plant by using peptone water medium in the concentration range of 1-120 mg/L has been reported by Mortazavis et.al in 2005. Two processes, adsorption on the cell surface and bioaccumulation have been observed. Maximum removal of mercury from the solution by *Pseudomonas putida* was found to be 98% (Mortazavis et. al, 2005).

Bioproducts such as chitosan, wool and peanut skins can be utilized for sorption of mercuric ion (Okino et. al, 2000). Scientists have reported accumulation of mercuric ion by genetically engineered *Escherichia coli JM109* expressing metallothionein and mercuric ion transport system (Okino et. al, 2000). *Chlorella* sp. has a high sorptive capacity for mercury and other metal ions due to complex mixture of sugars, uronic acids, glucosamine and proteins on its surface (Baldi et.al, 1993). Mercury adsorption by nonliving biomass of the brown marine macroalgae *Cystoseira baccata* was reported by Herrero et. al in

2005. The potential use of *Penicillum canescens* was reported for the removal of mercuric ion from aqueous solution (Say et. al, 2009). *Klebsiella pneumoniae* and *Pseudomonas aeroginosa* were also reported for mercury biosorption (Al-Garni et. al, 2010). The adsorption of mercury from aqueous solution by the use of fungal biomass of *Aspergillus versicolor* is also reported (Das et. al, 2007). In this context, iron bioprecipitation was investigated for mercury bioremoval from aqueous solution.

Materials and Methods

Screening of isolates

Selected three isolates obtained from different water and soil samples were studied for mercury bioremoval using 5 ppm mercury containing Casitone glycerol yeast autolysate medium (Appendix I).

Preparation of 100 ppm stock mercury solution

Analytical grade 13.5 mg mercury chloride was dissolved in 100 ml of deionised water, which gave the stock solution having 100 ppm mercury concentration. The working standard was prepared by appropriate dilutions from the stock solution.

Shake flask study of mercury removal

The mercury tolerance limit of two isolates, *Bacillus cereus* (GP_1) and *Enterobacter* sp. (S_4) were studied. Cultures were inoculated in 250 ml Erlenmeyer flask containing 50 ml of citrate broth medium, (Appendix I), supplemented with 5 and 10 ppm of mercury. Uninoculated flask in the experimental sets served as negative control. Flasks were incubated in orbital shaker (Newtronics, India) rotating at 150 rpm at 32±2 ºC temperature. After incubation the system was centrifuged at 9000 g for 20 min. Residual mercury was estimated from the supernatant by malachite green complex method (Appendix II). The growth was monitored in terms of optical density at 630 nm (Vogel, 1962).

Effect of organic media on mercury bioremoval

To find out the suitable medium for mercury removal, 250 ml Erlenmeyer flask containing 50 ml of nutrient broth or citrate broth or minimal medium or nutrient broth containing ferric ammonium citrate were studied. The composition of all the media is given in Appendix I. All the media were inoculated with 10% v/v actively growing culture of *Enterobacter* having 4.8 x 10^8 cells per ml. In all the flasks, 5.0 ppm of mercury was added. The flasks were incubated on environmental orbital shaker at 150 rpm and 32±2 ºC temperature. The samples were periodically removed and mercury removal was measured spectrophotometrically at 630 nm by malachite green complex method (Appendix II) (Vogel, 1962).

Effect of inoculum size on mercury bioremoval

Experiments were carried out in 250 ml Erlenmeyer flask containing 50 ml nutrient broth with or without ferric ammonium citrate (Appendix I) supplemented with 5 ppm mercury. All the flasks were inoculated with 5, 10, 15% v/v inoculum having 4.8x10^8 cells/ ml.

Effect of pH on mercury bioremoval

The pH of citrate broth medium was adjusted to 3.0, 5.0 and 7.0 with 0.1 N NaOH or 0.1 N HCl. The flasks were inoculated with *Enterobacter* sp. (S_4) inoculum having 4.2×10^8 cell/ml and it was incubated in orbital environmental shaker at 150 rpm and 32±2 ºC temperature. Control flask was setup without inoculation. Aliquots were taken periodically.

Effect of ferric ammonium citrate concentration on mercury bioremoval

To study the influence of ferric ammonium citrate concentration on mercury Bioremoval, 1, 0.1 and 0.01 g/L of ferric ammonium citrate was added in citrate broth (Appendix I). Total system of 50 ml was prepared containing 5 ppm of mercury and it was inoculated with 10% v/v inoculum of 4.5×10^8 cells/ml of actively growing *Enterobacter* sp. (S_4).

Influence of growing cells and harvested cells on mercury bioremoval

To check the influence of growing cells and harvested cells of *Enterobacter* sp. (S_4) on mercury bioremoval, Erlenmeyer flask containing 50 ml citrate broth and another flask containing 50 ml nutrient broth with ferric ammonium citrate was taken with 5.0 ppm mercury (Appendix I). Actively growing 10% v/v *Enterobacter* sp. (S_4) having 4.5×10^8 cells/ml was inoculated in the system. Mercury removal was estimated after 24 h. Further, 5.0 ppm of mercury was added in 24 h grown cells in each flask and kept in environmental orbital shaker for 15-20 min rotating at 150 rpm. Aliquots were taken and mercury removal was estimated by malachite green complex method (Appendix II).

Lab scale column reactor study

The glass column of 38 cm length and 3.6 cm outer diameter and 3.5 cm inner diameter was taken. Total volume of the column was 130 ml and working volume was kept 60 ml. Glass wool was inserted at

lower end of the column and filled with polystyrene beads as supporting material. An injector was inserted at the junction of pipe for introducing compressed air. Nutrient broth medium containing ferric ammonium citrate was inoculated with 10% active inoculum of *Enterobacter* sp. in the column with vigorous aeration. Synthetic waste water containing 5 ppm of mercury was added in the column. Samples were collected from the column at regular interval of time and were analysed for mercury removal. Configuration of column is shown in Table 25.

Table 25. Configuration of the column developed for mercury bioremoval

Configuration	Value/Specification
Vessel	Glass
Capacity (ml)	130
Working volume (ml)	50
Medium	Nutrient broth + FAC
Inoculum 10% v/v	4.2 X 10^8 cells/ml
Inner diameter (cm)	3.5
Outer diameter (cm)	3.8
Aeration (L/min)	0.5-1
pH	6.0
Inert support	Polystyrene beads
Bead diameter (mm)	3

Result and Discussion

Screening of isolates

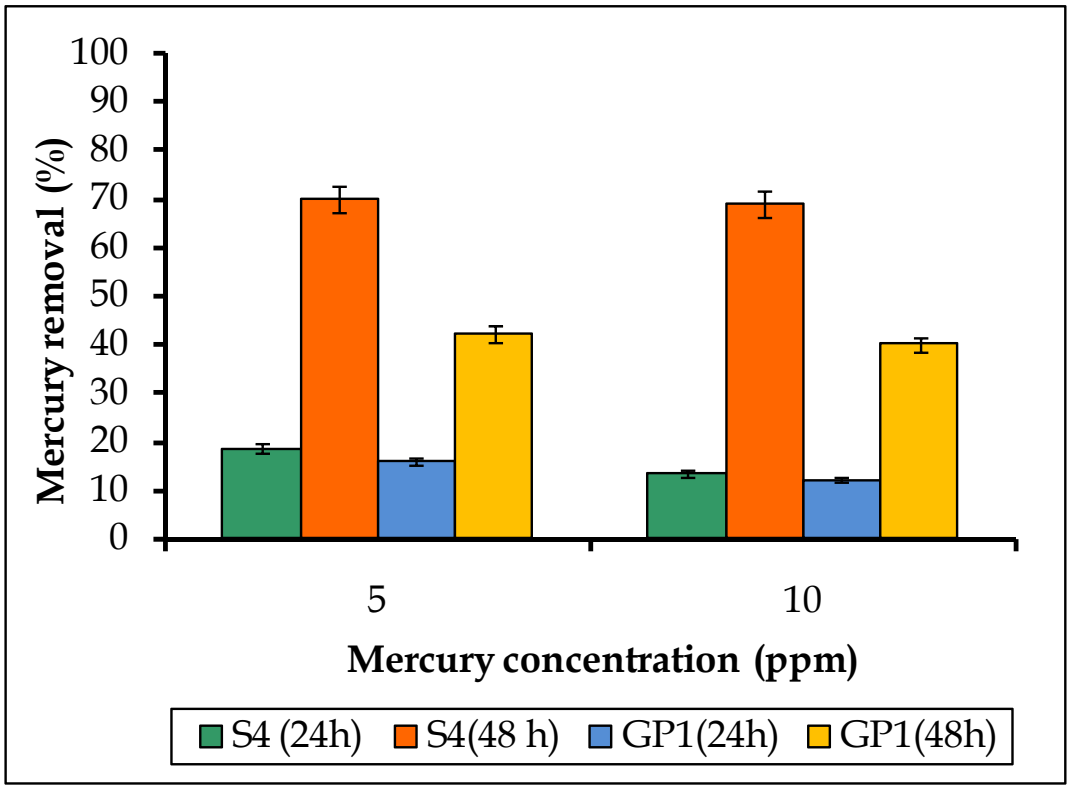
Mercury removal by selected three isolates is shown in Table 26. Isolates S_4, GP_1 and DI_2 showed 70%, 42% and 15% mercury removal respectively from the medium.

Table 26. Screening of isolates for mercury bioremoval

Isolates	Mercury removal (%)
S_4	70
GP_1	42
DI_2	15

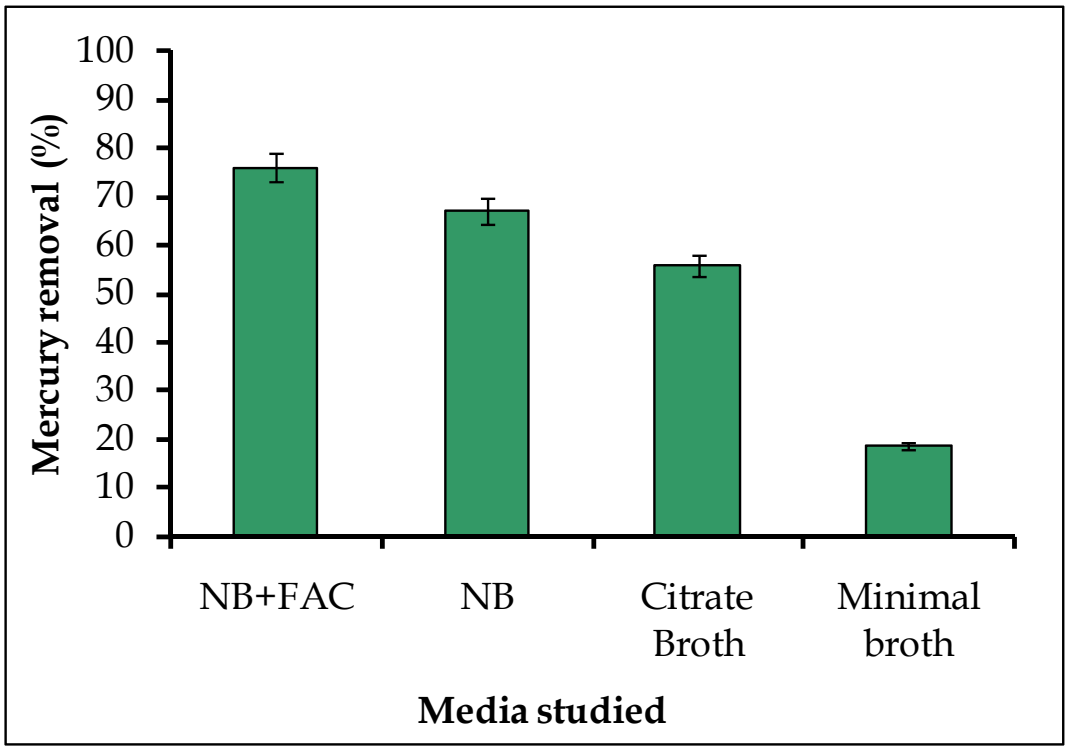
Shake flask study of mercury bioremoval in citrate broth was performed for *Enterobacter* sp. (S_4) and *Bacillus cereus* (GP_1) at 5 and 10 ppm of mercury concentration. The obtained results are shown in Graph 30. In 24 h of contact time irrespective of mercury concentration or the type of organisms used in study, hardly 20% of added mercury was found to be removed. In 48 h of contact time, the removal reached to as high as 70% by *Enterobacter* sp. (S_4) and 42% by *Bacillus cereus* (GP_1). *Enterobacter* sp. (S_4) was found to be more efficient for mercury bioremoval as compared to *Bacillus cereus* GP_1. Thus, all further studies were performed with *Enterobacter* sp.

Graph 30. Shake flask study of mercury bioremoval by selected isolates



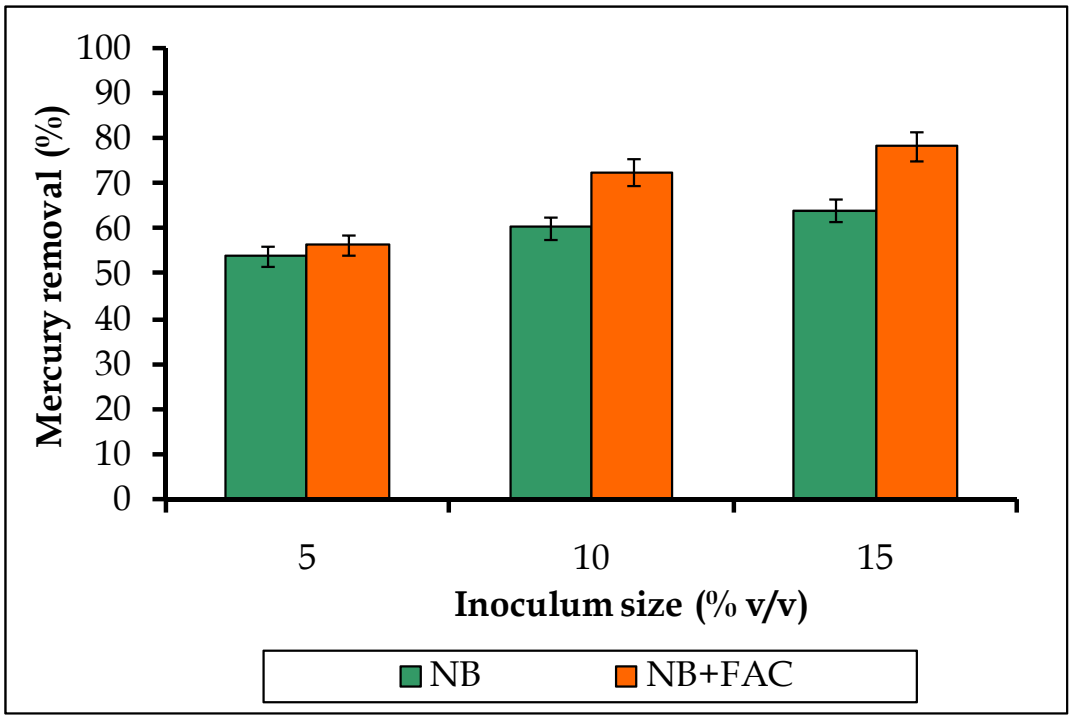
The influence of media composition on mercury bioremoval is shown in Graph 31. Presence of organic substrate in the medium showed higher mercury bioremoval as compared to mercury bioremoval in minimal broth. The combination of peptone in nutrient broth and ferric ammonium citrate (NB+FAC) resulted in the highest mercury removal, which comes out to be 76% as compared to mercury removal in nutrient broth and citrate broth. The presence of peptone in the medium enhanced the growth of organism and ferric ammonium citrate was precipitated due to microbial growth. The synergistic effect of peptone and ferric ammonium citrate could be responsible for the highest mercury removal in nutrient broth containing ferric ammonium citrate medium.

Graph 31. Mercury bioremoval in different media by *Enterobacter* sp. (S_4)



Influence of inoculum size on mercury bioremoval in nutrient broth and nutrient broth containing ferric ammonium citrate by *Enterobacter* sp. (S_4) is depicted in Graph 32. The mercury bioremoval was in direct proportion to the amount of inoculum added. It is obvious as higher the biomass higher the mercury bioremoval. *Enterobacter* sp. (S_4) showed the highest mercury bioremoval which was 79% in nutrient broth containing ferric ammonium citrate.

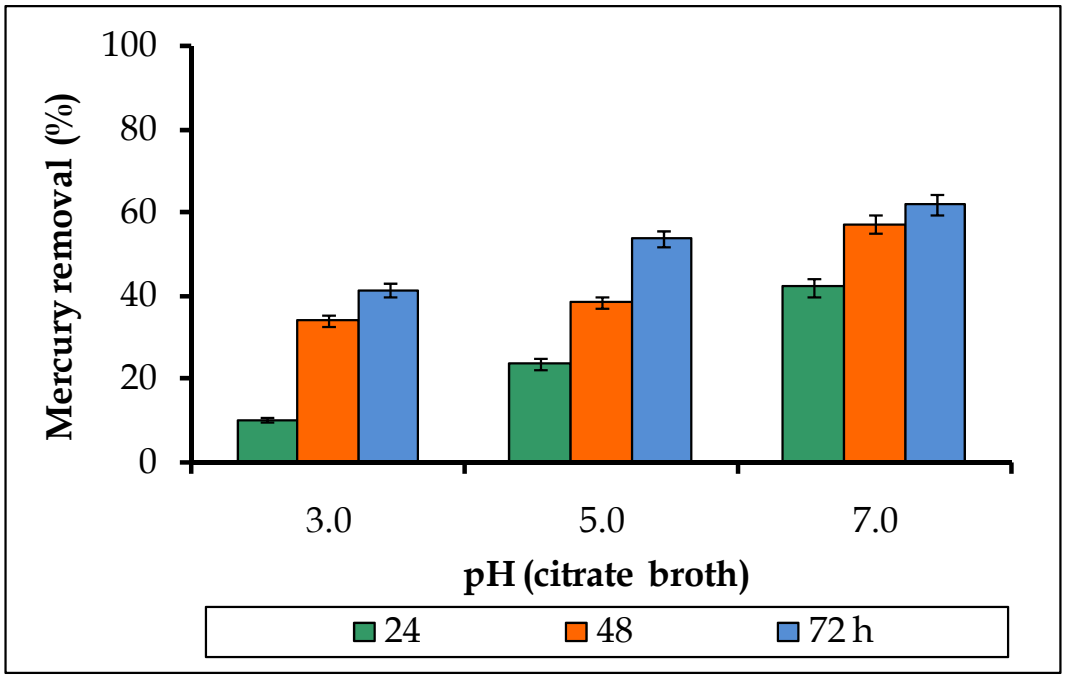
Graph 32. Influence of inoculum size in NB and NB + FAC on mercury bioremoval by *Enterobacter* sp. (S_4)



Influence of pH on mercury bioremoval is shown in Graph 33. As the pH was raised towards alkaline sides the mercury bioremoval also increased. This could be due to increase in precipitation of ferric ammonium citrate at neutral pH as well as better growth of *Enterobacter* sp. (S_4) at this pH as compared to both these activities at pH 3.0 and pH 5.0. Mercury removal enhanced with increase in medium pH. Medium pH affects the solubility of metal ions and ionization state of the functional groups. At low pH values, cell surface being positively charged would not be favourable for the attachment of positively charged mercuric ion due to repulsion. It can also be explained as low amount of metal ion retained by the biosorbent at pH value below 4, because most functional group expected to dissociate only at neutral pH values. The increase in

biosorption level observed with increasing pH can be explained by strong relation of biosorption to the number of surface negative charges, which depends on the dissociation of functional groups. It is observed that adsorption of mercury increases with increasing pH values. The high adsorption believed to be associated with the formation of positively charged metal hydroxyl species, having strong affinity for surface functional group (Okoronkwo et. al, 2007).

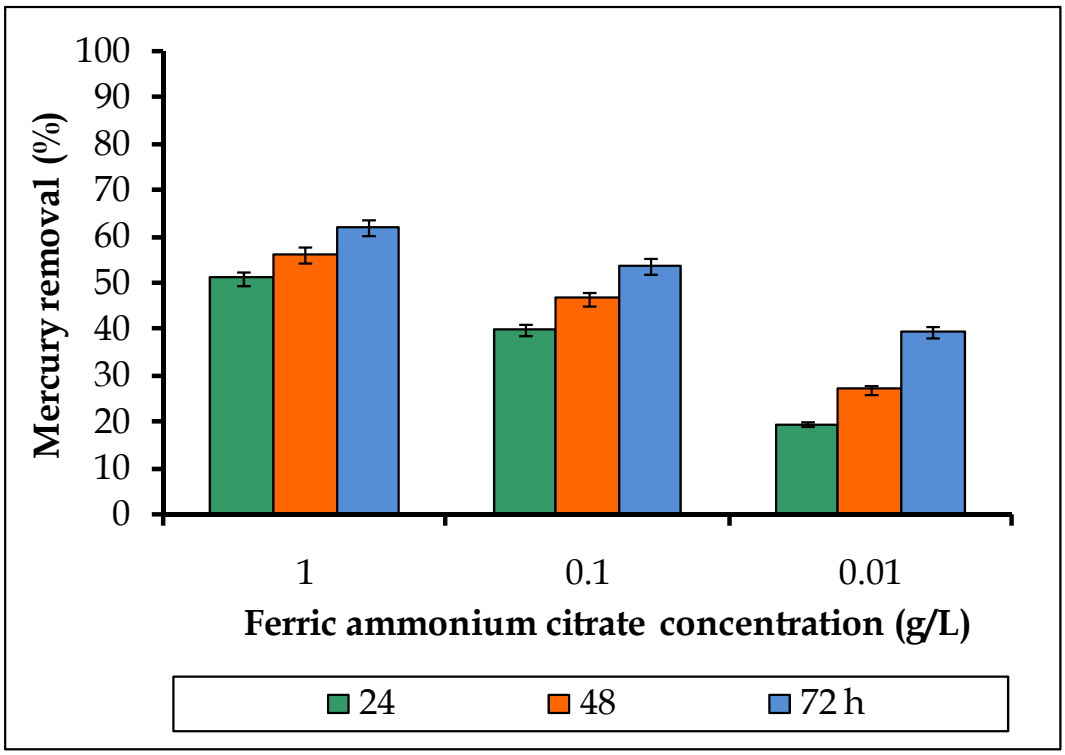
Graph 33. Influence of pH of citrate broth on mercury bioremoval by *Enterobacter* sp.



Effect of ferric ammonium citrate (FAC) in the range of 0.01 to 1 (g/L) in citrate broth on mercury bioremoval by *Enterobacter* sp. was studied and results are shown in Graph 34. The mercury bioremoval was in direct proportional to ferric ammonium concentration in the medium. However, 1 g/L FAC resulted in heavy precipitation in the medium. Thus, 0.1 g/L ferric ammonium citrate was considered to be

optimum in terms of mercury removed, amount of precipitate formed and decolourization of the medium.

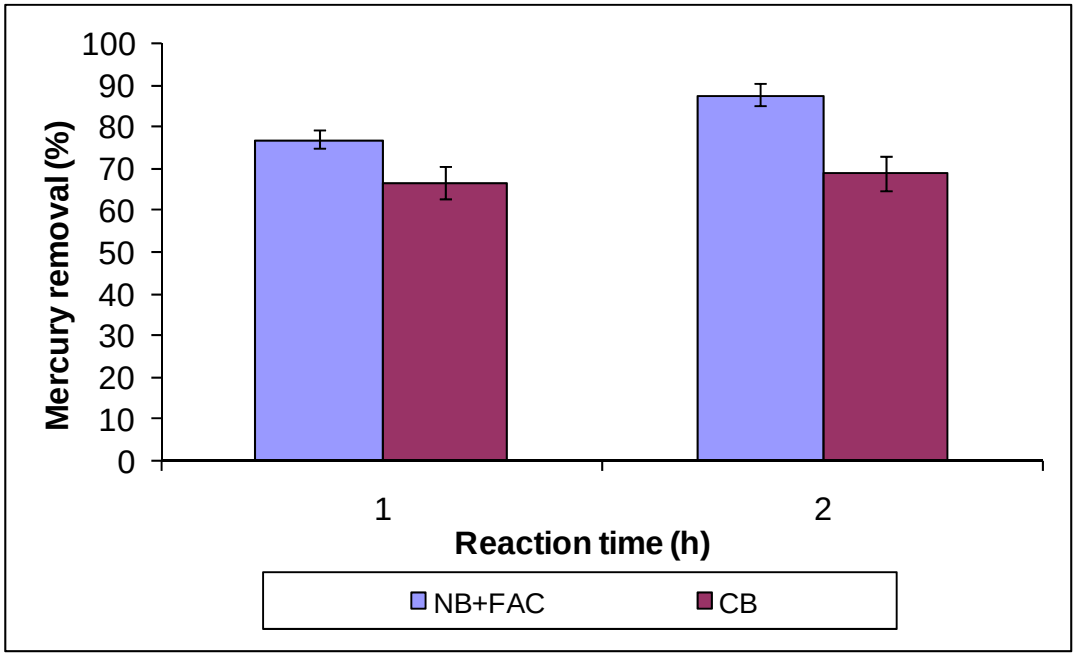
Graph 34. Influence of FAC concentration on mercury bioremoval by *Enterobacter* sp. (S_4)



Experiment was also conducted to elucidate influence of growing cells and harvested cells on mercury bioremoval in nutrient broth containing ferric ammonium citrate (NB+FAC) and citrate broth by *Enterobacter* sp. (S_4) and results are shown in Graph 35. The mercury bioremoval was almost similar in case of mercury added in the beginning and when it was added at the end of 24 h of growth. Thus it indicated that metabolites produced by biomass and change in the medium composition due to microbial growth could be responsible for mercury removal. Thus, mercury can be removed efficiently even by the organism grown in the nutrient broth containing ferric

ammonium citrate (NB+FAC) or citrate broth medium in 15-20 min of contact after 24 h of growth. Thus, this method could be used for higher concentration of mercury in the system without any adverse effect of mercury toxicity on the inoculum.

Graph 35. Influence of growing cells and harvested cells on mercury bioremoval by *Enterobacter* sp. (S_4)



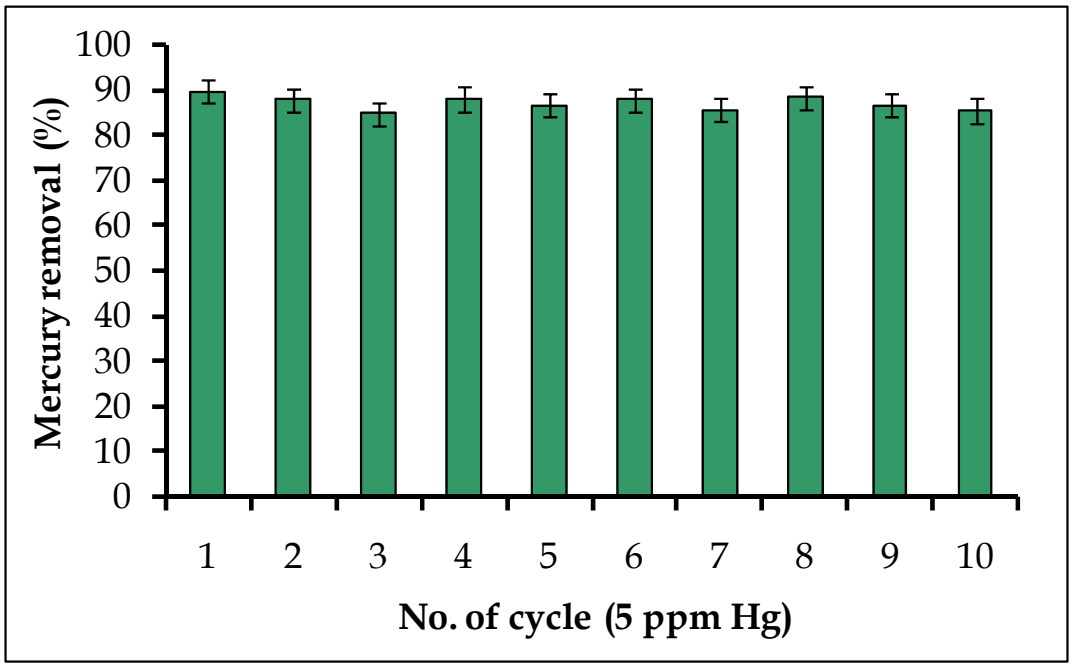
1. Hg was added at the end of 24 h of growth.
2. Hg was added with the inoculum and allowed to react for 24 h

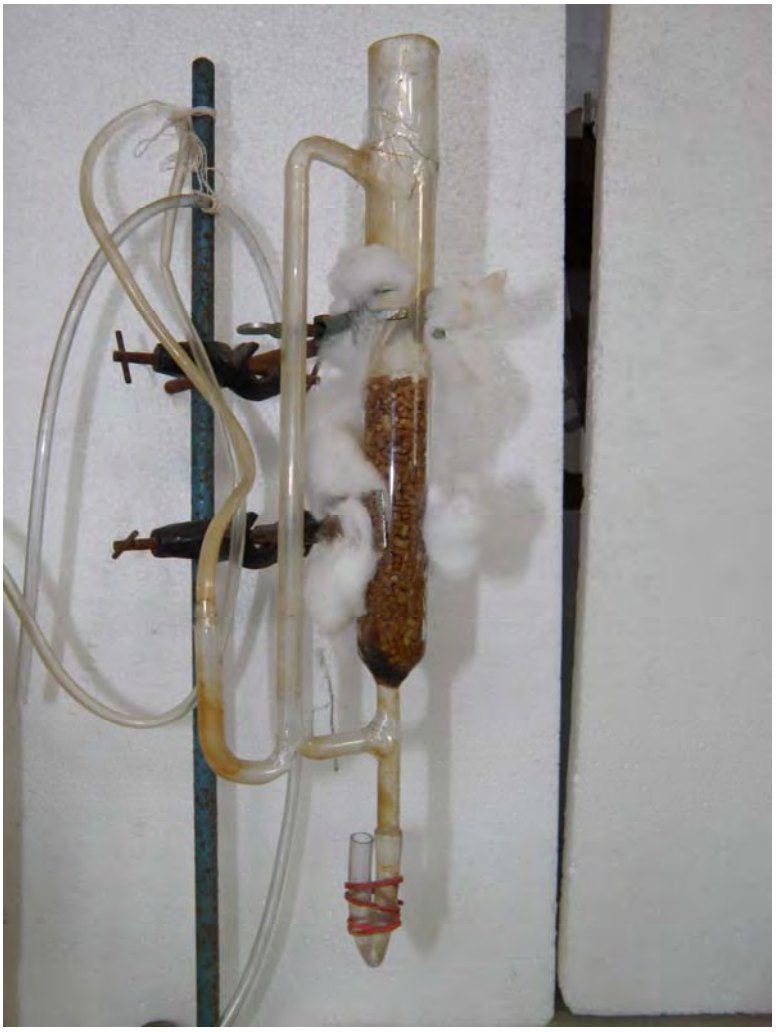
Laboratory scale column study

After 10 cycles of addition of nutrient broth containing ferric ammonium citrate inoculated with *Enterobacter* sp. (S4), biomass was found to be deposited on polystyrene beads and accumulated in the column. This indicated development of biofilm. The results obtained by this immobilized biomass for mercury bioremoval from 5 ppm mercury containing synthetic waste is shown in Graph 36. As can be

seen from the result, in all the ten cycles, mercury bioremoval was 88±2% in 18 h of contact time. The picture of lab scale column reactor used in the study is shown in Photograph 13.

Graph 36. Lab scale column reactor study of mercury bioremoval by *Enterobacter* sp. (S4)





Photograph 13. Laboratory scale column reactor for mercury bioremoval

Chapter 6

Cadmium Bioremoval

Introduction

Industrial discharge containing cadmium is strictly controlled due to the high toxic nature of this element and its tendency to accumulate in the tissue of living organism (Tilaki and Ali, 2003). Cadmium is usually found at quite low concentrations in crystal rocks (Santra, 2005). Naturally a very large amount of cadmium is released into environment through weathering of rocks, through forests fires and volcanic eruptions (http://www.lenntech.com /periodic/elements/ cd.htm).

Properties

Cadmium is soft, bluish white metallic element occurring primarily in zinc, copper and lead ores. The density of cadmium is 8.65 gm/cm^3. Cadmium is bivalent metal. It is similar in many respects to zinc but reacts to form more complex compounds. The most common oxidation state of cadmium is +2, though in rare case +1 can be found. Naturally occurring cadmium is composed of eight isotopes (http://www.answers.com/topic/cadmium?). In combination with certain metals, cadmium makes some common low melting point alloys such as woods metal and Abel's metals. Cadmium reacts slowly with oxygen in moist air at room temperature. Cadmium does not react with water, though it reacts with most acids (http://www.chemistryexplained.com/). Properties and image of cadmium is shown in Table 27 and Figure 12.

Table 27. Properties of cadmium

Property	Value
Atomic number	48
Atomic mass (g/mol)	112.4
Electro negativity according to Pauling	1.7
Density (g/cm^3 at ºC)	8.7
Melting point (ºC)	321
Boiling point (ºC)	765
Vanderwaal's radius (nm)	0.154
Ionic radius (nm)	0.097 nm (+2)
Energy of first ionization kJ/mol	866
Energy of second ionization kJ/mol	1622
Isotopes	15
Standard potential	-0.402 V
Discovered	Fredrich Stromeyer, 1817

Data adapted from http://www.lenntech.com/periodic/elements/cd.htm

Figure 12. Cadmium mineral image (http://library.thinkquest.org/C0113863/gfx-bin/Cadmium.jpg)

Occurrence

The only important ore of cadmium is green ockite or cadmium sulphide (http://en.wikipedia.org). The major producers of cadmium are Canada, USA, Mexico, Japan Peru and Australia (http://www.lenntech.com/periodic/elements/cd.htm).

Applications

Cadmium is widely used in pigments as heat stabilizer for plastics, for corrosion resistance of steel and cast iron, metal plating, phosphate fertilizer, mining and refining processes, pigments, alloy industries, in soldering and brazing, for production of certain pesticides, for production of X-ray screens and nickel-cadmium battery manufacturing industry (Mahvi and Bazrafshan, 2007). It is used for colouring pigment production which is used in fabrics, textiles, paints etc (Santra, 2005). Cadmium is used as barrier to control neutrons in nuclear fission (http://en.wikipedia.org). Cadmium oxide is used in black and white television as phosphors and also as blue and green phosphors for colour television picture tubes. Cadmium telluride can be used for light detection or in solar cells (http://www.answers.com/topic/cadmium?).

Sources of pollution

Cadmium is released from various industrial activities such as mining, smelting and electroplating etc. and its high toxicity makes it necessary to remove it from source of pollution of the biosphere (Macaskie and Dean, 1984). The major sources of cadmium in human are cigarette smoking, certain foods such as shell fish, coal

burning and contaminated water. Coal burning routinely generates cadmium because coal contains substantial amount of cadmium. The coal power plant usually generates waste in the form of huge ash or bottom ash. Cadmium can be removed from waste water through ferric sulphate coagulation at a pH above 8.0 through lime softening. The cadmium ions are precipitated as hydroxide at pH 10.0 or 11.0. Combustion of fossil fuels, roasting and smelting of ores, kiln operation in cement industry and incineration of wastes release cadmium into the environment. Cadmium vapour is emitted from processes in the form of fugitive emission or through flue gas system. (http://www.cpcb.nic.in/oldwebsite).

Cadmium toxicity

The U.S. department of health and human service has reported that there is sufficient evidence in human for the carcinogenicity of cadmium and cadmium compound given by agency for toxic substances and diseases registry (ATSDR) toxicology profiles, 1999. The toxic effects of cadmium are associated with metal affinity for organic ligands containing sulphur, nitrogen or other electronegative functional groups. Cadmium has no known useful role in higher organisms. The most dangerous form of occupational exposure to cadmium is inhalation of fine dust and fumes or ingestion of highly soluble cadmium compounds (http://en.wikipedia.org). Cadmium interactions with micronutrients and other dietary components are shown in Table 28.

Table 28. Cadmium interactions with micronutrients and other dietary components (http://ehp.niehs.nih.gov/members/1998/ Suppl-1/203-216peraza/perazatab1.GIF)

Metal		**Toxicity**
Cd		Anemia; csteoporosis; proximal tubular disfunction leading to hypertension, coronary arteies disease and chronic pulmonary diseases
Metal-nutrient	**Interaction and mechanism**	**Effect of nutrient on metal toxicity**
Cd- zinc	Competes for GI absorption; Cd interferes with zinc metabolism	Reverses Cd toxicity (i.e. decreases growth, increases lesions and testicular necrosis)
Cd- iron	Cd decreases iron absorption and metabolism (Cd possibly binds with ferritin and transferring)	Supplementation corrects anemia: increases hematocrit and increases haemoglobin levels
Cd- copper	Cd interferes with copper metabolism possibly by decreasing copper absorption	Corrects Cd induced decreased plasma ceruloplasmin concentrations
Cd- protein	Low protein diet results in increased Cd uptake	Sufficiency prevents Cd induced decreased growth, decreases MT synthesis and increases bone deformities
Cd- selenium	Selenium shifts Cd binding to higher molecular weight proteins	MT can now bind essential nutrient

1. Cadmium acts directly on the kidney to enhance sodium and water retention. It can cause hypertension and proteinuria (http://www.arltma.com/).
2. Loss of calcium from bones leads to multiple bone fractures and high alkaline phosphates in blood. Cadmium toxicity also causes high rise in blood pressure and other heart diseases (Santra, 2005).

   In 1955, the occurrence of a mysterious disease in the Jintsu basin of Japan near the city of Toyama is characterized as **Itai-Itai** or **Ouch-Ouch** disease. The symptoms of this disease are severe pain in back, joints and lower abdomen. Development of waddling or duck like gait (Santra, 2005).

3. Damage to immune system, central nervous system and cancer development. (http://www.lenntech.com/periodic/elements/cd.htm).
4. Cadmium can adversely affect the elasticity of lung tissue and causes fatal lung damage (http://www.arltma.com/).
5. Excess cadmium causes a number of toxic symptoms in plants like inhibition of photosynthesis, altered stomatal action, induction and inhibition of enzymes, efflux of cations and generation of free radicals (Prasad, 1995).

6. Low level of cadmium causes nausea, vomiting and diarrhoea. Inhaled cadmium dust causes dryness of throat, headache and pneumonia like symptoms (http://www.chemistryexplained.com). Toxicity of cadmium is shown in Figure 13.

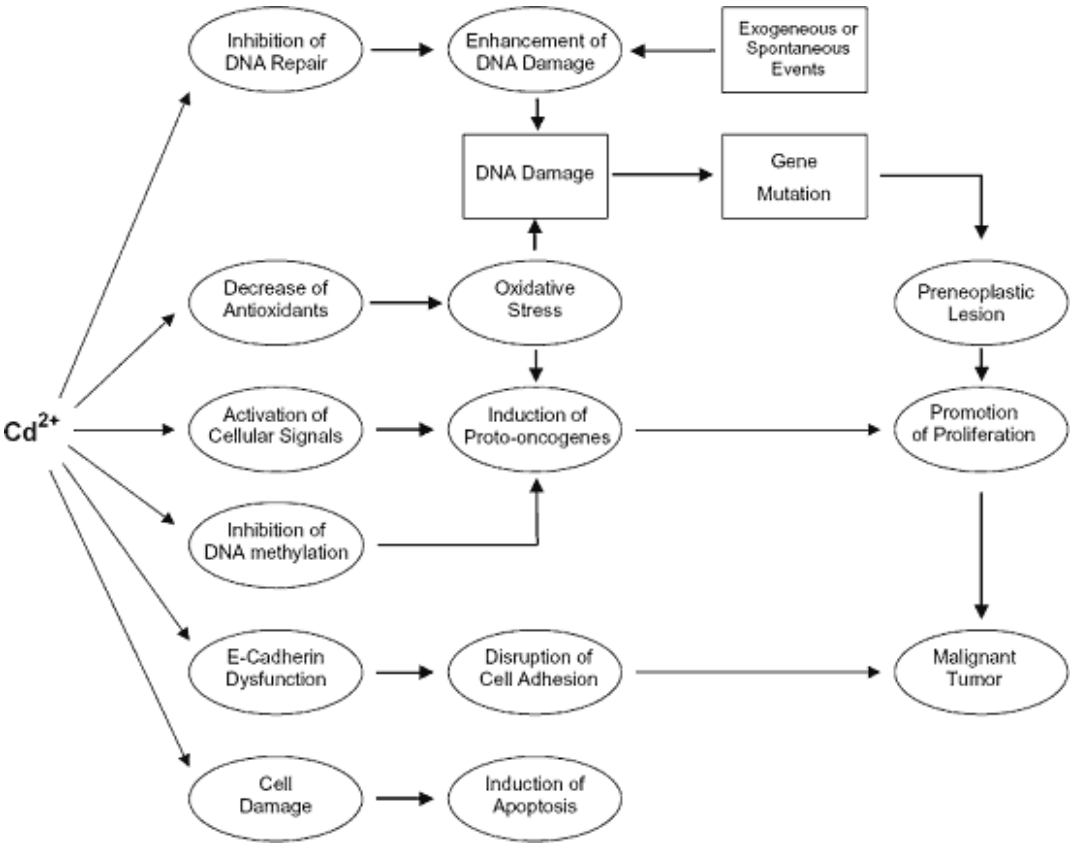


Figure 13. Cadmium toxicity (http://www.bioconcepts.com.au /images/newspic/cadmium_chart.gif)

Conventional methods for removal of cadmium

Removal of cadmium from effluents, before they are discharged into the environment, can be accomplished by processes such as chemical precipitation, cementation, solvent extraction and ion exchange. These processes are sometime neither selective nor effective and some of them are very expensive (Chatterjee, 2006). The technology for removing cadmium from industrial waste water or from flue dust is well established (Santra, 2005). In waste water, dissolved cadmium can be precipitated with sodium sulphide, cemented by the addition of zinc or separated out by ion exchange.

If the cadmium is incorporated into particulates, it can be dissolved by addition of acid and then separated by one of the above techniques or the solids can be setteled out and the cadmium is removed with the sludge (Santra, 2005). Disadvantages of physicochemical processes are expensive, high reagent requirement and generation of toxic sludge.

Bioremoval of cadmium

Biosorption using microbial biomass as an adsorbent has emerged as a potential technique for metal removal (Talos et. al, 2009). Cadmium uptake by different Gram positive organisms like S*taphylococcus,* B*acillus subtilis*, Gram negative bacteria like *Pseudomonas aeruginosa* and yeast *Candida utilis* has been reported (Wang et. al, 1997; Kujan et. al, 2006). A fluorescent *Pseudomonad* CW- 96-1 strain isolated from deep sea vent sample grew at 30 ºC under aerobic condition in an artificial seawater medium containing citrate and tolerated cadmium concentration up to 5 mM. After 140 h of inoculation, strain CW- 96-1 removed 99% of cadmium from solution. Energy dispersive microanalysis revealed that the cadmium was removed by precipitation on the cell wall (Wang et. al, 1997). *Citrobacter* sp. isolated from lead polluted soil, was found to be resistant to cadmium and accumulated the metal when grown in its presence (Macaskie and Dean, 1984). Both photosynthetic bacterium *Rhodovulum* sp. and *Rhodobacter sphaeroides* are capable of cadmium removal (Watanabe et. al, 2003). Dead biomass of *Actinomycetes,* which is the waste product from industrial fermentation, was mixed with waste water as free

bacterial suspension and biosorption occurred. Cadmium cation bound to negative charged sites on bacterial cell wall and could be desorbed from the cell wall when needed (Chatterjee , 2006).

Various metal oxides and hydroxides have been extensively explored and are still being worked upon for their sorbent property. One such important sorbent is ferric hydroxide which binds trace elements and wide range of metals and metalloid like arsenic, selenium, cobalt, nickel, cadmium and zinc (Chakravorty and Van Grieken, 1986). Biosorption of lead, copper, zinc and cadmium onto *Sphaerotilus natans* at different equilibrium pH (3.0 – 5.0) were reported and the pollutant uptake is reported to increase with increase in pH (Pagnanelli et al., 2003).

Non living and dried biomass of *Paecilomyces variotii* and *Cladosporium resinae* fungi were used for the removal of cadmium from aqueous solution in batch mode or shake flask condition. Biosorption of Cd^{2+} to non living biomass of *Rhizopus arrhizus* and *Schizomeris leiblenii* were studied in batch reactor. The optimum pH was found to be 5 for maximum adsorption rate of Cd^{2+} ions. The adsorption rate increases with increase in cadmium concentration for organisms upto 100-150 mg/ml respectively. The adsorption by *Rhizopus arrhizus* were higher than that of *Schizomeris leiblenii* (Chatterjee , 2006)).

Biomass of *Candida utilis* biomass can conveniently be used for cadmium biosorption from aqueous solution (Kujan et al., 2006). The Cd^{2+} ion adsorption on native *Saccharomyces cerevisiae* biomass of different origin in aqueous suspension was studied. The

biosorbents were commercial baker's yeast from Hungary, waste yeast from brewery (Cluj, Romania and Pecs, Hungary) and cultivated *saccharomyces cerevisiae* fungal cells. The cultivated yeast proved to be the best sorbent for cadmium removal. The least cadmium amount was adsorbed by waste yeast from brewery of Cluj, Romania (Talos et. al, 2009).

Biosorption of cadmium by biomass of dry brown marine alga, *Sargassum polycystum* was investigated in batch system. High cadmium uptake capacity and abundant availability of *Sargassum polycystum* indicated that it can be used for the development of biosorbent for heavy metal removal from waste water (Srikrajib et al., 1999).

Adsorption processes using agricultural waste products is becoming the new alternative for waste water treatment. The effectiveness of adsorption of cadmium ion by sugarcane bagasse was studied by determining the maximum adsorption capacity of cadmium by batch mode process. The high adsorption was achieved at agitation rate of 150 rpm and pH range of 5.0-7.0. Cadmium removal also increases with increasing pH of the solution (Ibrahim et al., 2006). In this context, iron bioprecipitation was investigated for its role in cadmium bioremoval from aqueous solution.

Materials and method

1. Screening of isolates

Selected three isolates obtained from diversified ecosystems were studied for cadmium bioremoval, using 10 ppm of cadmium containing casitone glycerol yeast auytolysate medium (Appendix I).

2. Preparation of stock solution

Stock solution of cadmium (100 ppm) was prepared by dissolving 22.8 mg of cadmium sulphate octahydrate in acidified water of pH 5.5. The working standard was prepared by appropriate dilutions from the stock solution.

3. Shake flask study of Cadmium bioremoval

Bacillus cereus and *Enterobacter* sp. were evaluated for cadmium bioremoval. These experiments were carried out in 250 ml Erlenmeyer flask at 30±2 ºC temperature with a working volume of 50 ml of citrate broth supplemented with 10 and 20 ppm of cadmium and inoculated with actively growing 10% v/v culture of *Enterobacter* sp. or *Bacillus cereus,* having 4.2 $\times 10^8$ cells/ml. Experiments were performed along with negative control simultaneously. Flasks were incubated in environmental orbital shaker (Newtronics, India) rotating at 150 rpm. At specific interval of times, samples were collected and centrifuged at 9000g for 15 minutes. Appropriate dilution was prepared by using acidified water and cadmium analysis (Appendix II) was carried out by using atomic absorption spectrophotometer (Elico India, model SL 191).

4. Cadmium bioremoval in different organic media

To access the effect of different medium on cadmium bioremediation, experiments were conducted in 250 ml Erlenmeyer flask containing 50 ml of total system of nutrient broth, nutrient broth containing ferric ammonium citrate and citrate broth (Appendix I) respectively. In all the media 10 ppm of cadmium was added. Flasks were inoculated with 10% v/v inoculum having 4.2×10^8 cells/ml. Uninoculated flask was kept as a negative control. Flasks were incubated in orbital shaker rotating at 150 rpm and 30±2°C temperature. Samples were withdrawn after regular interval of time and centrifuged at 9000 g for 10 minutes. The supernatant was diluted as per requirement and remaining cadmium was estimated by atomic absorption spectrophotometer (Elico India, model SL -191).

5. Effect of pH on cadmium bioremoval

The effect of pH on metal bioremoval was studied in the range of 3.0 to 7.0. The pH of medium was adjusted using 0.01 N HCl or 0.01 N NaOH, prior to addition of inoculum. Actively growing culture of *Enterobacter* sp. was inoculated in the system having 4.3×10^8 cells/ml. Flasks were incubated on environmental orbital shaker at 150 rpm and 30±2 °C temperature. At regular time interval, 5 ml of culture broth was removed and centrifuged to remove the biomass. The residual metal concentration was determined after appropriately diluting the supernatant.

6. Influence of inoculum size on cadmium bioremoval

The effect of varying inoculum size of 5, 10 and 15% v/v medium on cadmium bioremoval was studied. Flask containing total system of 50 ml citrate broth supplemented with 10 ppm of cadmium and inoculated with actively growing *Enterobacter* sp. having 4.2×10^8 cells/ml. Experiments were carried out along with appropriate control that were run simultaneously. Flasks were incubated in orbital shaker at 150 rpm and 30±2 ^oC temperature. Samples were withdrawn after regular interval of time and centrifuged at 9000g for 10 min. The supernatant was diluted for cadmium analysis by atomic absorption spectrophotometer (Elico India, model SL-191).

7. Effect of ferric ammonium citrate concentration on cadmium bioremoval

The effect of ferric ammonium citrate (FAC) concentration on cadmium bioremoval was studied. In the system of 50 ml citrate broth, 10%(v/v) of inoculum of actively growing *Enterobacter* sp. having 4.2×10^8 cells/ml was used to inoculate the medium. The amount of 1 and 0.1 g/L of FAC was added in citrate broth and agitated at 150 rpm and 30±2^oC temperature. The supernatant was diluted for cadmium analysis by atomic absorption spectrophotometer (Elico India, model SL-191).

8. Influence of growing cells and harvested cells on cadmium bioremoval

To check the influence of growing cells and harvested cells of *Enterobacter* sp. on cadmium bioremoval, Erlenmeyer flask containing 50 ml citrate broth and another flask containing 50 ml nutrient broth was taken with 10 ppm cadmium. Actively growing 10% v/v *Enterobacter* sp. having 4.2×10^8 cells/ml was inoculated in the system. Cadmium removal was estimated after 24 h. Further, 10 ppm of cadmium was added in 24 h grown cells in each flask and kept in environmental orbital shaker for 15-20 min at 150 rpm. Residual cadmium was estimated by atomic absorption spectrophotometer (Elico India, model SL-191).

Results and Discussion

Screening of isolates

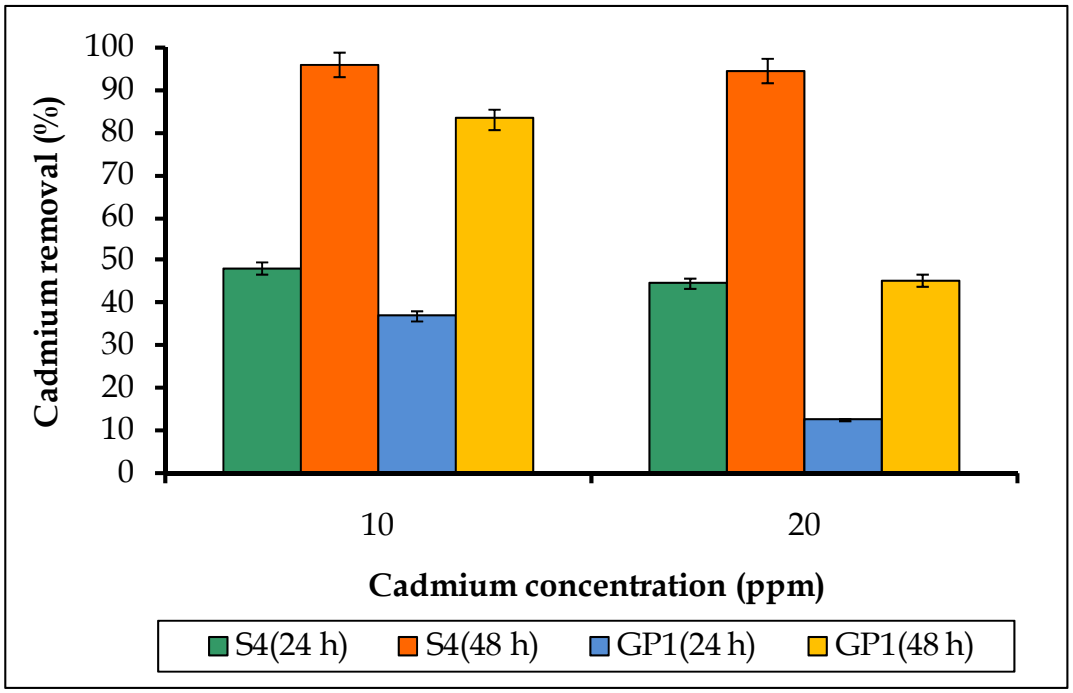
Cadmium removal by selected three isolates is shown in Table 29. Isolates S_4, GP_1 and DI_2 showed 95%, 82% and 9% cadmium removal respectively from the medium.

Table 29. Screening of isolates for cadmium bioremoval

Isolates	Cadmium removal (%)
S_4	95
GP_1	82
DI_2	9

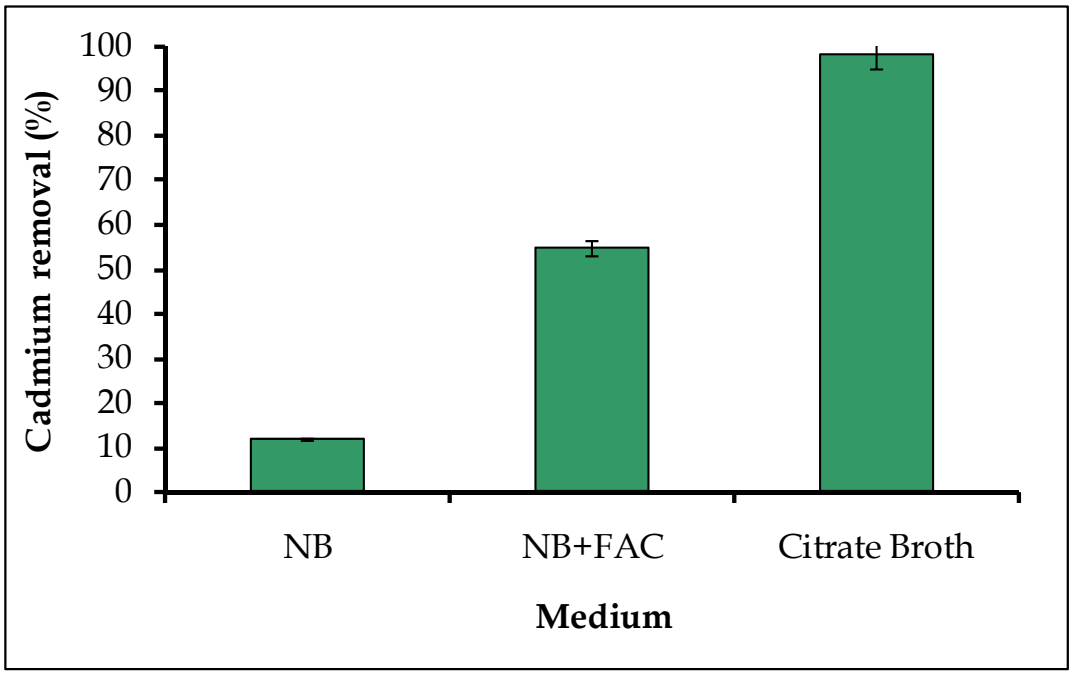
Shake flask study for cadmium bioremoval in citrate broth by *Enterobacter* sp. (S_4) and *Bacillus cereus* (GP_1) at 10 and 20 ppm of cadmium concentration was performed. The obtained results are shown in Graph 37. *Enterobacter* sp. has better cadmium removal ability as compared to *Bacillus cereus*. In *Enterobacter* sp. there was marginal difference in cadmium removal efficiency when cadmium concentration was increased from 10 to 20 ppm. In 48 h of contact time *Enterobacter* sp. showed as high as 95±2% cadmium removal irrespective of concentration used. Whereas two fold reduction in cadmium removal was observed in similar condition in case of *Bacillus cereus.* Thus, all other study was performed with *Enterobacter* sp.

Graph 37. Shake flask study of cadmium bioremoval by selected isolates



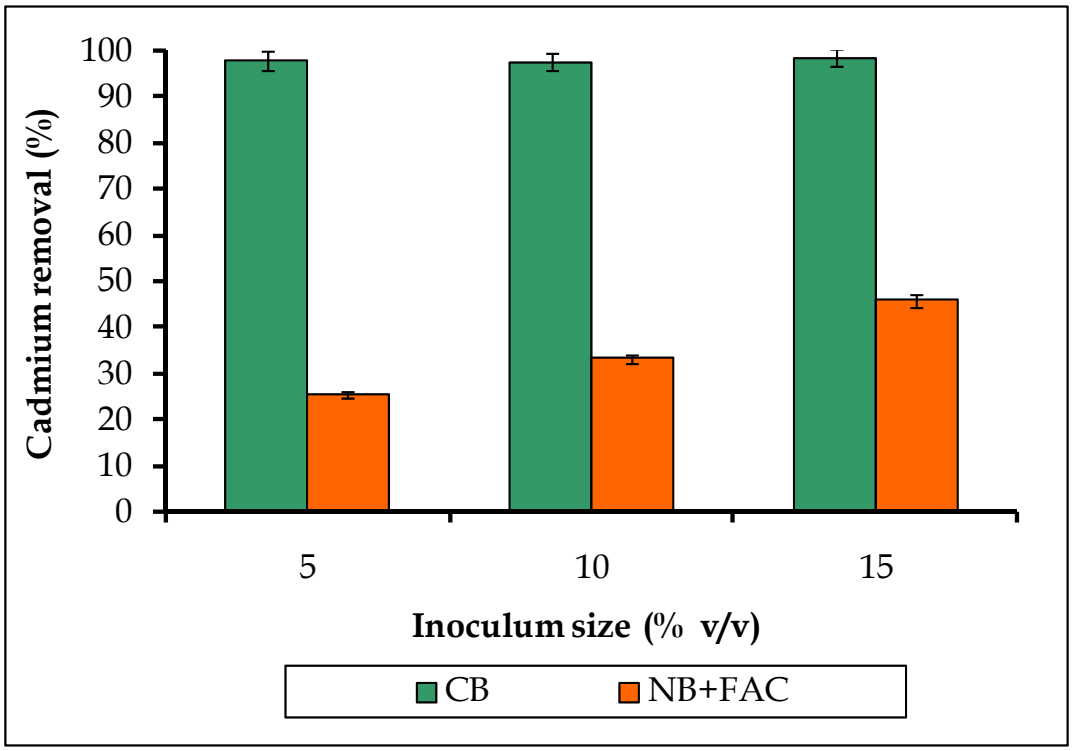
The influence of media composition on cadmium bioremoval by *Enterobacter* sp. is shown in Graph 38. The highest cadmium removal of 98% was observed in citrate broth as compared to nutrient broth and nutrient broth containing ferric ammonium citrate (NB+FAC).

Graph 38. Cadmium bioremoval in different organic media by *Enterobacter* sp.



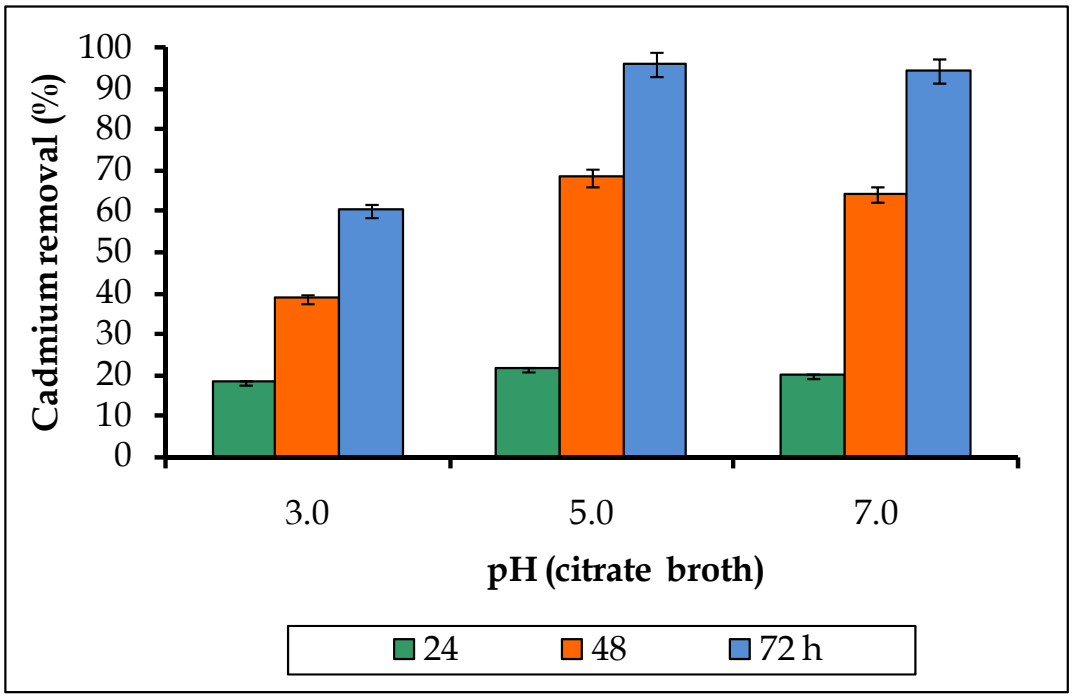
Influence of inoculum size on cadmium bioremoval in citrate broth and nutrient broth containing ferric ammonium citrate by *Enterobacter* sp. is depicted in Graph 39. The cadmium bioremoval was in direct proportion to the amount of inoculum added. It is obvious due to higher biomass which resulted in higher cadmium bioremoval. *Enterobacter* sp. showed highest cadmium bioremoval as high as 97% in citrate broth as compared to nutrient broth containing ferric ammonium citrate.

Graph 39. Influence of inoculum size in citrate broth and NB+FAC on cadmium bioremoval by *Enterobacter* sp.



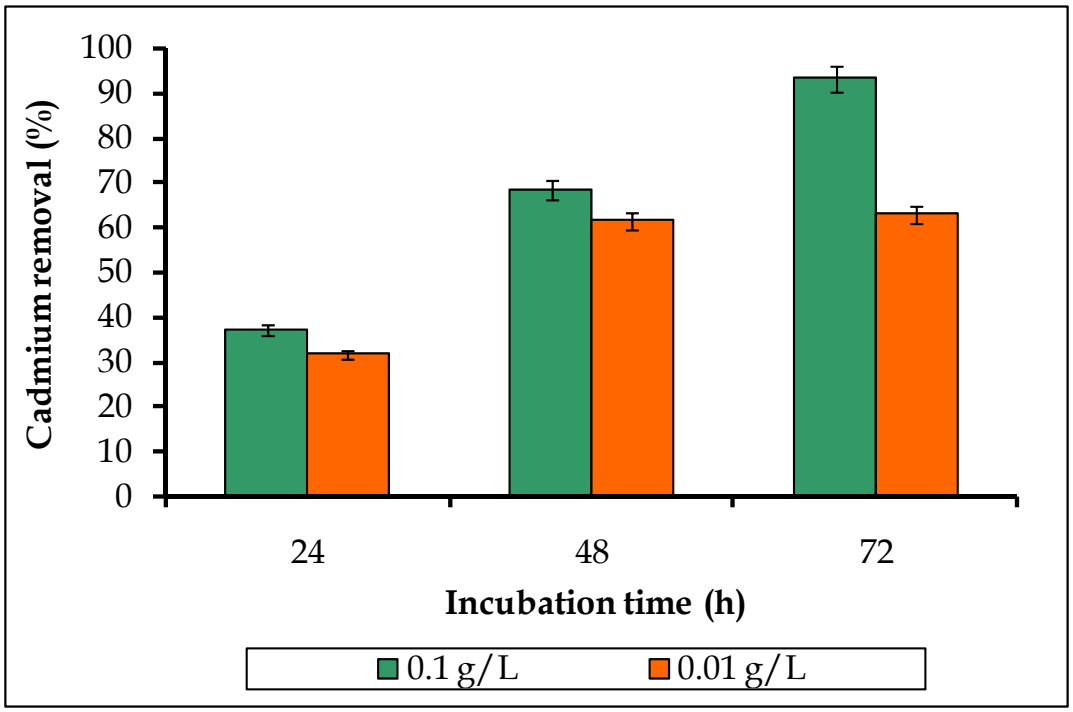
Influence of pH on cadmium bioremoval is shown in Graph 40. As the pH was increased towards alkaline side the cadmium bioremoval also increased. This could be due to increased precipitation of ferric ammonium citrate at neutral pH, thus cadmium removal was better at pH 5.0 and 7.0. pH is one of the most important controlling parameters in all adsorption processes. At low pH, cadmium ions had to compete with H^+ ions for adsorption sites on the adsorbent surface. As the pH increased this competition weakens and more cadmium ions were able to replace H^+ ions to the adsorbent surface (Ibrahim et. al, 2006).

Graph 40. Influence of pH of citrate broth on cadmium bioremoval by *Enterobacter* sp.



Effect of ferric ammonium citrate (FAC) in the range of 0.1 to 1.0 g/L in citrate broth was studied on cadmium bioremoval by *Enterobacter* sp. and results are shown in Graph 41. The cadmium bioremoval was in direct proportional to ferric ammonium citrate concentration in the medium. However, 1 g/L ferric ammonium citrate (FAC) resulted in heavy precipitation in the medium. Thus, 0.1 g/L ferric ammonium citrate (FAC) was considered to be optimum in terms of cadmium removed, amount of precipitate formed and decolourization of the medium.

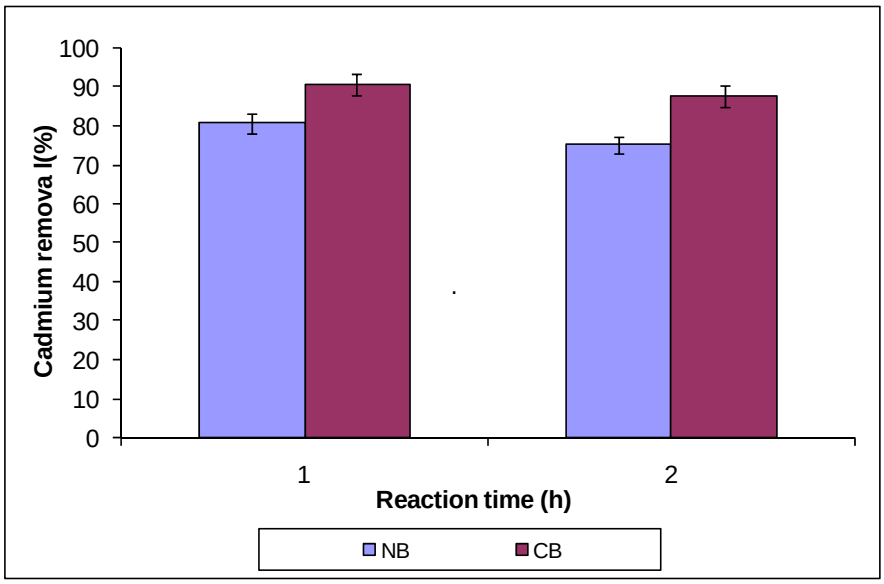
Graph 41. Cadmium bioremoval at different FAC concentrations by *Enterobacter* sp.



As can be seen from the result given in Graph 42, the cadmium bioremoval was almost similar in case of cadmium added in the beginning and when it was added at the end of 24 h of growth. Thus it indicates that metabolites produced by biomass and change in the medium due to microbial growth could be responsible for cadmium removal. So, cadmium can be removed efficiently even by the organism grown in the nutrient broth or citrate broth medium in 15-20 minute of contact after 24 h of growth. Therefore, this method could be used for higher concentration of cadmium in the system without any adverse effect due to the toxicity of cadmium on growth of test organisms. Lee (1975) described that high sorption tendency of ferric hydroxide exists primarily during the oxidation of reduced iron to ferric hydroxide precipitate.

Freshly precipitated ferric hydroxide is amorphous and it has the ability to sorb (incorporate into the precipitate) heavy metals and many organics. In fact, this property of ferric hydroxide is used in several wastewater treatment processes such as for the removal of phosphate, selenium and dissolved organic carbon. Martinez and Mc Bride in 2000 investigated the precipitation of several heavy metals with ferric hydroxide. They reported that copper, cadmium, lead and zinc were coprecipitated with ferric hydroxide and the binding of the metal in the ferric hydroxide depended on the type of metal (Lee, 2005).

Graph 42. Influence of growing cells and harvested cells on cadmium bioremoval by *Enterobacter sp.* (S4)



1. Cd was added at the end of 24 h of growth.
2. Cd was added with the inoculum and allowed to react for 24 h

Chapter 7

Selenium Reduction

Introduction

Properties

Selenium is a member of chalcogen family. It is a metalloid element similar to sulphur and tellurium in chemical activity and physical properties (http://www.chemistryexplained. com).

Selenium combines with hydrogen, fluorine, chlorine and bromine. It burns in presence of oxygen with bright blue flame to form selenium dioxide. Isotopes selenium-75 is used to study the function of pancrease and parathyroid gland (http://www.chemistryexplained. com). Selenium exists in several allotropic forms. The most thermodynamically stable and dense form of selenium is electrically conductive gray (trigonal form) which is composed of long helical chains of selenium atoms (http://en.wikipedia.org/wiki). Three deep red monoclinic forms are alpha, beta and gamma. Amorphous red selenium and black vitreous selenium are used for industrial purpose (http://www.chemistryex plained.com/A-r/Allotropes. html). Red amorphous form originates when Se^0 precipitates in aqueous solution. At temperature greater than 30 ºC, red form changes to black (Shukla, 2009). Selenium occurs in four valence states: selenates (Se^{6+}), selenites (Se^{4+}), selenides (Se^{2+}) and elemental selenium (Se^0) (Ehrlich, 1981). Properties and image of selenium is shown in Table 30 and Figure 14.

Table 30. Properties of selenium

Property	Value
Atomic number	34
Atomic mass (g/mol)	78.96
Electro negativity according to Pauling	2.4
Density (g/cm^3 at 20ºC)	4.79
Melting point (ºC)	217
Boiling Point (ºC)	688
Vanderwaal's radius (nm)	0.14
Ionic radius (nm)	0.198 nm (-2) ; 0.042 nm (+6)
Electronic shell	[Ar] 3d10 4S2 4P4
Energy of first ionization kJ/mol	940.7
Energy of second ionization kJ/mol	2045
Energy of third ionization (kJ/mol)	2973.7
Standard potential	-0.77 V
Discovered	Jons Berzelius ,1817

Data adapted and modified from (http://www.lenntech.com/periodic/elements/se.htm)

Figure 14. Image of selenium mineral

(http://www.galleries.com/minerals/elements/selenium/selenium.jpg)

Selenate

Selenate is analogous to sulfate. They are highly soluble in aqueous solutions at ambient temperature and are highly mobile. It can be reduced to selenite and selenium (http://en.wikipedia.org/wiki/ selenate). Bioremediation of selenate contaminated drainage water has been demonstrated in pilot studies using selenate respiring bacterium *Thauera selenatis* (Shukla, 2009).

Selenites

It is prepared via the neutralization of selenious acid (H_2SeO_3) by the oxides, hydroxides or carbonates of the corresponding metals. It is highly mobile. It forms two series of salts: normal selenites and acid or hydroselenites (Carroll, 1999).

Selenide

Selenium forms selenides with metals e.g aluminum selenide, mercury selenide, lead selenide (http://en.wikipedia.org/ wiki). Selenide is a reduced form of selenium, a gas which is highly toxic and is rapidly oxidized (Shukla, 2009). Alkali, alkaline earth's heavy metals all can form selenides. Heavy metal selenides are insoluble in water (Carroll, 1999).

Sources of pollution

Selenium is distributed throughout the environment by processes including volcanic activity, rock and soil weathering, leaching of soils, uptake and release by plants, animals and microorganisms, chemically and biologically mediated oxidation reduction reactions and mineral formation. Anthropogenic activities such as burning of fossil fuels and disposal of industrial effluents and agricultural drainage water also redistribute selenium in the environment (Carroll, 1999). According to NAS (1976) and USEPA (1984), the largest anthropogenic sources of atmospheric selenium are from the combustion of fossil fuels. The production and refining of copper particulates are the primary expected form of the compound (http://oehha.ca.gov/air/ chronic_rels/pdf/selenium.pdf).

Occurrence

The nations producing selenium include the United states, Belgium, Canada, Chile, Germany, Japan and Sweden (http://www.eoearth.org/article/selenium).

Function

It is essential in very small amount for health of both plants and animals. Selenium is naturally present in grains, cereals and meat. Human being need to absorb certain amount of selenium daily in order to maintain good health (http://www.lenntech.com/periodic /elements/se.htm). Selenium combines with proteins to aid in the creation of antioxidants, the regulation of some thyroid function and healthy functioning of the immune system (http://www.wisegeek. com).

Uses of selenium

1. Selenium compounds are used in the glass industry as decolorizing agent and used in rubber industry as vulcanizing agents. It is also used in toning baths, photography and xerography and in insecticides. Selenium sulphide is used in shampoo as an anti-dandruff agent (http://oehha.ca.gov /air/chronic_rels/pdf/selenium.pdf).
2. Selenium is used in photocopying, photocells, light meters and solar cells because of its photovoltaic and photoconductive properties (http://en.wikipedia.org/wiki).
3. Selenium is used in metal alloys such as the lead plates used in storage batteries and in rectifiers to convert AC current to DC current (http://www.lenntech.com/periodic/elements/se.htm).

Toxicity

Selenium is an essential trace element but it is toxic if taken in excess. Selenium toxicity also called selenosis (http://www.wisegeek.com /what-is-a-selenium-toxicity.htm). Selenium uptake through food may be higher when selenium rich fertilizers have been applied on farm land. Due to irrigation run off concentration of selenium tend to be very high in aquatic organisms in many areas. Selenium from hazardous waste sites and from farm land will end up in ground water or surface water through irrigation (http://www.Lenntech .com/periodic/elements/se.htm). The selenium toxicity symptoms are as follows:

1. Gastrointestinal disorders.
2. Hair loss.
3. Sloughing of nails.
4. Garlic odour in breath.
5. Fatigue, irritability, neurological disorders and liver cirrhosis.
6. Acute occupational exposure to selenium dioxide resulted in bronchospasm (accumulation of fluid in lungs and bronchitis may occur), irritation of the upper respiratory passage, violent coughing, nausea and vomiting.
7. Skin rashes and decay of teeth.
8. Sore throats, fever, shortness of breathe, conjunctivitis and abdominal pain.
9. A serious problem occurred at the Kesterson reservoir in Northern California. In 1970s, scientists found that birds nesting in the reservoir were developing genetic deformities. They traced the

problem due to high level of selenium in water (http://oehha.ca .gov/air/chronic_rels/pdf/selenium.pdf; http://en.wikipedia. org/; http://www.lenntech.com; http://www.chemistryexplaine d .com).

Conventional methods

Conventional methods for removing selenium contaminants from water include chemical addition followed by precipitation or adsorption to a solid phase or membrane filtration to separate the oxidized or reduced selenium species. U.S Patent no. 4,915,928 describes a process for removing selenium from waste water using a strong ion exchange resin (http://www.faqs.org/patents). Other methods for selenium remediation are iron co-precipitation, membrane ultrafilteration, electro dialysis, reverse osmosis (Carroll, 1999). Conventional methods for removing selenium are ineffective or extremely expensive due to the existence of salt, especially sulphate in the majority of selenium polluted water (Soudi et. al, 2003).

Biological methods

Biological transformation of selenium pollutants by plants and microbes has been considered as an alternative (De souza et. al, 2001). Microbial transformation is based on reduction of inorganic forms of selenium i.e. selenite and selenate by certain microorganisms and their conversion to elemental selenium or volatilization of selenium (Soudi et. al, 2003). Microorganisms involved in such bioremediation processes belong to the genera *Aeromonas, Arthrobacter, Acinetobacter,*

Wolinella, Pseudomonas, Sulfurodospirillum, Enterobacter, Bacillus and *Citrobacter* under oxic and anoxic condition. The bacterial reduction of selenite to elemental selenium by *Bacillus* sp. and *Pseudomonas fluorescence* has also been reported and confirmed by TEM (Belzile et.al, 2006).

Reduction of selenite was evidenced by the formation of red crystalline or amorphous precipitate in media containing selenium and lack of any precipitate in control flask. A maximum specific uptake rate for selenite of 3,040 μg Se^{4+}/g.cells/h is reported for *Shewanella putrefaciens* (Carroll, 1999). Biogeochemical cycling of Se in aquatic ecosystem is shown in Figure 15.

Acidithiobacillus ferrooxidans has also been reported to oxidize copper selenide (CuSe) to cupric copper (Cu^{2+}) and elemental selenium (Se^0) (Ehrlich, 1981). *Desulfovibrio desulfuricans* DSM 194 can be adapted to grow in presence of 1 μM selenate or 100 μM selenite. Reduction by *Desulfovibrio desulfuricans* was 95% and 97% respectively. As observed under electron microscopy with energy dispersive X-ray analysis, selenate and selenite were reduced to elemental selenium, which occurs inside the cell. Selenium granules resulting from selenite metabolism were cytoplasmic while granules of selenium, resulting from selenate reduction, appeared to be in the periplasmic region. After lysis of microbial cell by selenium toxicity, red elemental selenium granules get liberated in the media (Tomei, 1995). In this context, selenium reduction was studied by using iron precipitating cultures.

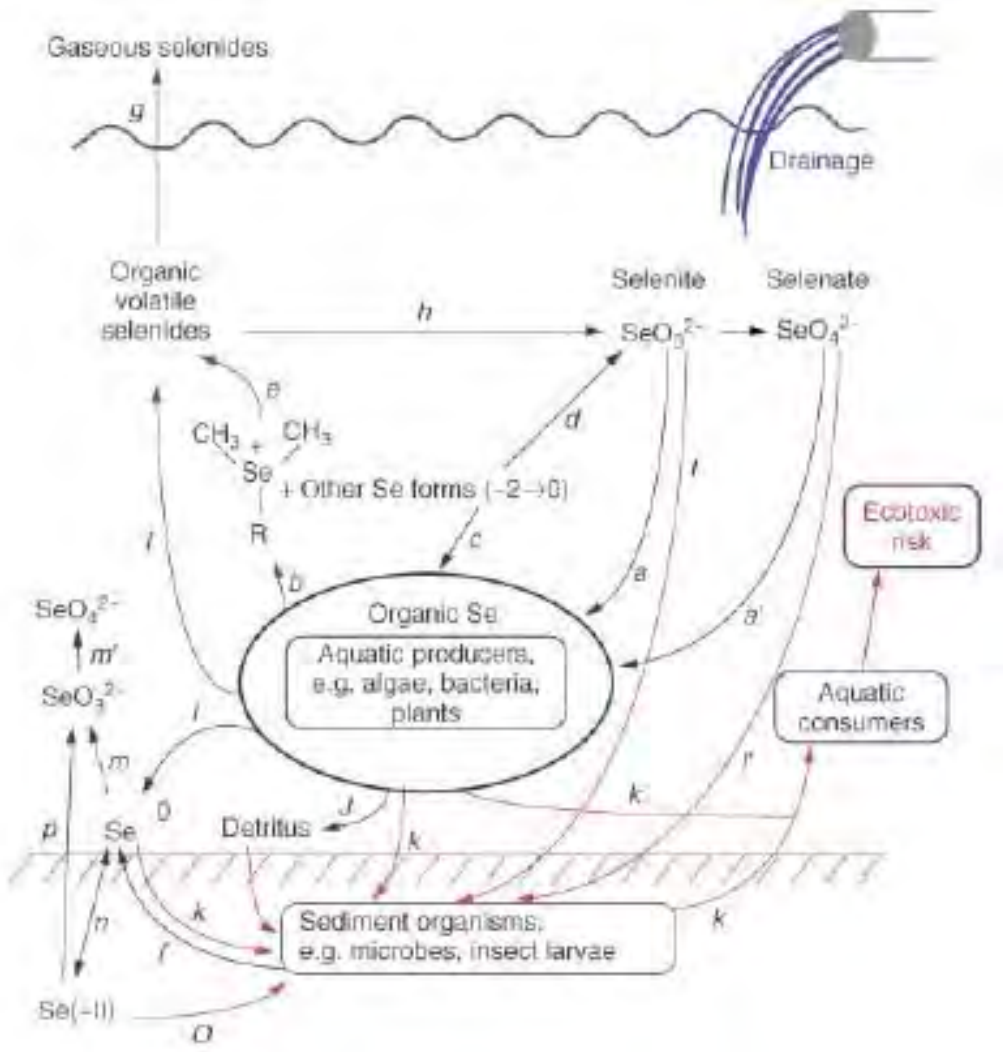


Figure 15. Biogeochemical cycling of Se in aquatic ecosystem.

Arrows indicate processes that can lead to risk from foodweb accumulation of Se ("ecotoxic" risk). Other arrows trace the Se volatilization process by which Se can be lost from the aquatic system (Higashi et. al, 2005)

Materials and Method

Screening of isolates

Selected three isolates obtained from different water and soil samples were studied for selenite reduction. All the isolates were streaked on casitone glycerol yeast autolysate (CGY) plates (Appendix I) containing 50 mM selenite. Plates were incubated at 30±2 ºC for 3 days and colonies were observed for reduction of selenite.

Medium for selenite reduction

Experiments were performed in 250 ml capacity Erlenmeyer flask with total system of 100 ml of casitone glycerol yeast autolysate (CGY) broth (Appendix I). The pH of media was adjusted to 6. Actively growing cultures was inoculated in the system and 1 M sodium selenite stock solution was added to the broth at a dosage of 5 ml and 10 ml to give final concentration of 50 mM and 100 mM respectively in the medium. Negative control for each test was kept without inoculation. Flasks were incubated in orbital environmental shaker (Newtronics, India) at 150 rpm at 30±2 ºC temperature. Aliquots were taken periodically and centrifuged at 9000g for 15 min (Remi India, C24)) and Se (IV) estimation (Appendix II) was done from the supernatant.

Results and Discussion

Screening of isolates

Reduction of selenite by selected three isolates is shown in Table 31. Isolate GP_1 showed 85% selenite reduction, while isolates S_4 andDI_2 showed 41 and 21% selenite reduction respectively. Hence, selected three isolates were identified as selenium reducers based on the ability to reduce selenite to elemental selenium. Red colonies formed by *Bacillus cereus* (GP_1) is shown in Photograph 14 and cell count of selenite reducers is given in Table 32.

Table 31. Screening of isolates for selenite reduction

Isolates	Selenite reduction (%)
GP_1	85
DI_2	21
S_4	41

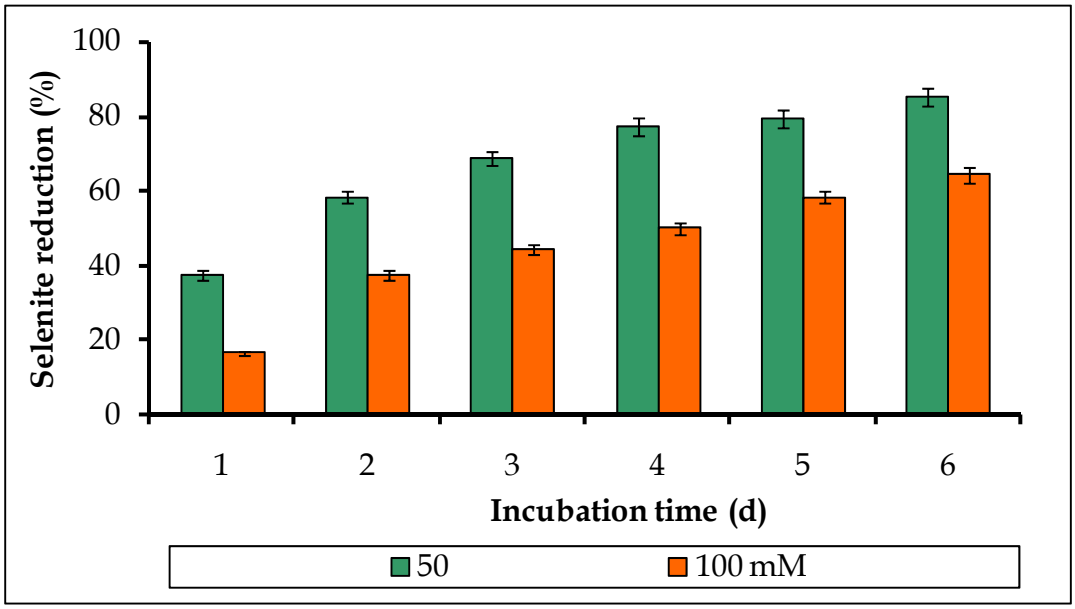
Photograph 14. Growth of *Bacillus cereus* (GP_1) on CGY Plate indicating formation of red coloured colonies

Table 32. Cell count of selenite reducer isolates

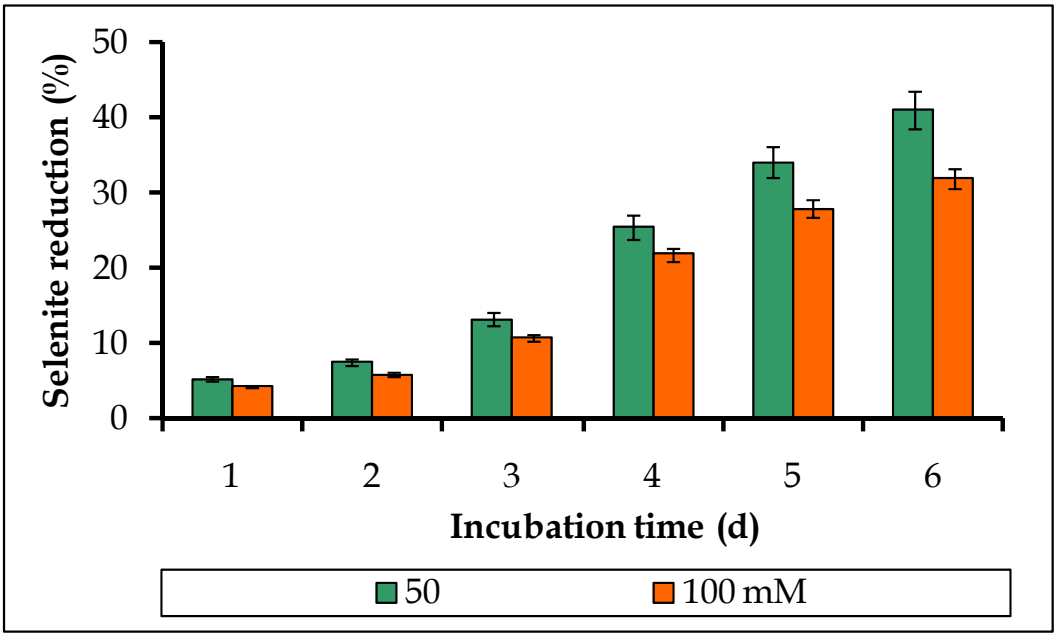
Sr. No.	Isolate	Cell count (10^6 cells/ml) (50 mM Selenite)
1	GP_1 (*Bacillus cereus*)	1.6
2	S_4 (*Enterobacter* sp.)	1.2
3	DI_2 (*Bacillus licheniformis*)	1

In quantitative studies at 50 mM selenite in the medium, isolate GP_1 - *Bacillus cereus* (Graph 43), isolate S_4 - *Enterobacter* sp. (Graph 44) and isolate DI_2 - *Bacillus licheniformis* (Graph 45) were found to grow well in CGY broth incorporated with selenite. Reduction of 50mM and 100 mM selenite was 85.2% and 64.3% by *B. cereus*, 41.1% and 32% by *Enterobacter* sp. after 6 days of incubation. *B. licheniformis* reduced 50mM selenite up to 21% only in 6 days.

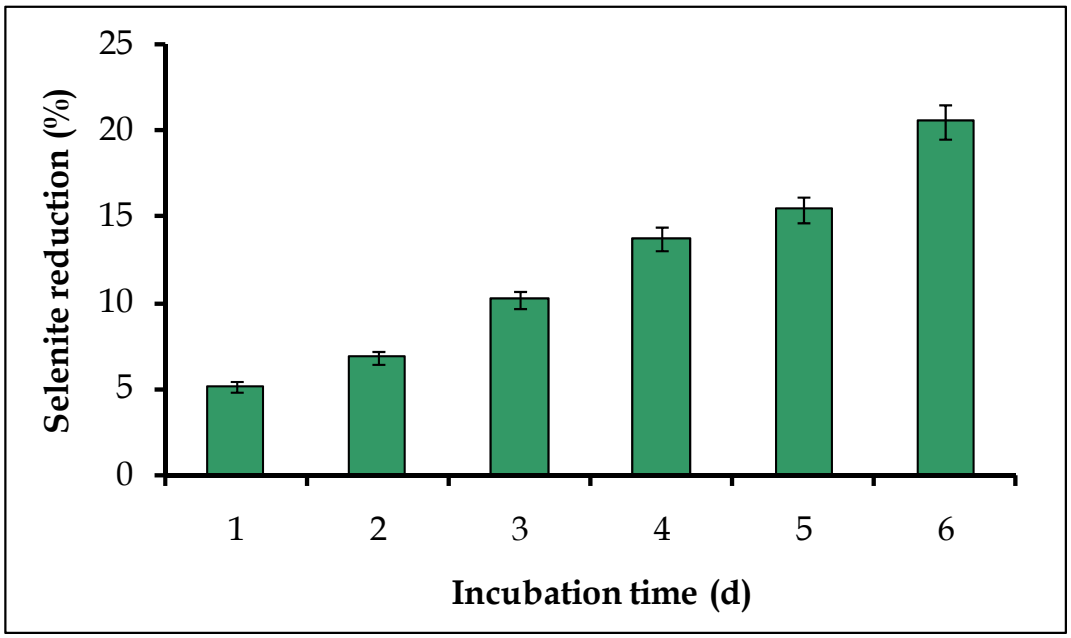
Graph 43. Selenite reduction by *Bacillus cereus* (GP_1)



Graph 44. Selenite reduction by *Enterobacter* sp. (S_4).



Graph 45. Selenite reduction by *Bacillus licheniformis* (DI_2)



The flasks containing bacteria and selenite showed the formation of red colouration and precipitates in the media over the course of 6 d. In control flask formation of red colouration was not observed. The growth of *Bacillus cereus* (GP_1) at 50 and 100 mM is shown in Photograph 15 (A) and (B).

A B

Photograph 15. The growth of *Bacillus cereus* (GP_1) at 50 and 100 mM selenite in CGY broth

The formation of red precipitation proved that selenite was reduced to elemental selenium. This is a two step process. Selenite is possibly reduced to Se^{2+} and eventually to red amorphous granules of elemental selenium. Hence, element selenium is the logical product of the selenate reduction. It has been reported that the Se^0 particle formed by the Se respiring bacteria *Sulfurospirillum barnesii, Bacillus selenitreducens* are structurally unique as compared to elemental selenium formed by chemical synthesis. *Rhodobacter sphaeroides* is a purple non sulphur bacterium can tolerate high concentration of selenite or selenate and can reduce or methylate these compounds (Shukla , 2009).

Chapter 8

Manganese Oxidation

Introduction

Properties

Manganese is the fifth most abundant metal in the earth's crust and is an essential trace element for all organisms (Depalmo, 1993). Manganese is a gray white, hard brittle metal. The metal tarnishes on exposure to air and when heated, oxidizes to Mn^{2+} oxides (http://www.chemicool/element/man.html). Pure manganese exists in four different allotropes (http://www.azom.com/details. asp?ArticleID=1699). Manganese is distinctive for being able to exist in a great number of oxidation states, from 0 to +7. Manganous cation (Mn^{2+}) is the most soluble form of manganese in nature. The +3 oxidation state is unstable and usually reverts to +2 state. Mn^{3+} and Mn^{4+} are found as insoluble oxides or hydrous oxides - Mn^{4+}, most notably as MnO_2. These oxides are brown or black coloured (Depalmo, 1993). Image of manganese and its properties are shown in Figure 16 and Table 33.

Figure 16. Image of manganese mineral
(http://0.tqn.com/d/chemistry/1/0/D/Q/manganese.jpg).

Table 33. Properties of manganese

Properties	Value
Atomic number	25
Atomic mass (g/mol)	54.93
Electro negativity according to pauling	1.5
Density (g/cm^3 at 20 ºC)	7.43
Melting point (ºC)	1247
Boiling point (ºC)	2061
Vanderwaal's radius (nm)	0.126
Ionic radius (nm)	0.08 nm (+2) ; 0.046 nm (+7)
Energy of first ionization (kJ/mol)	716
Energy of second ionization (kJ/mol)	1489
Energy of third ionization (kJ/mol)	2973.7
Standard potential	-1.05 V (Mn^{2+}/Mn)
Discovered by	Johann Gahn

Data adapted and modified from http://www.lenntech.com/periodic/elements /mn.htm

Occurrence

The main mining area for manganese ores are South Africa, Russia, Australia, Gabon, Brazil and India (http://enviornmentalchemistry. com).

Sources

Manganese minerals are widely distributed. Oxides, silicates and carbonates are the most common forms. (http://periodic.Lanl.gov /element s/25.html). Pyrolusite and rhodochrosite are the most common manganese bearing minerals. In addition to these sources many large nodules of manganese have been found on ocean floors that could provide another source of manganese (http://www.azom.com/).

Functions

1. Manganese activates enzymes like oxidoreductase, transferase, arginase and isomerase. It also plays an important role in the metabolism of carbohydrate, amino acids and cholesterols (http://www.chelationtherapyonline.com/articles/).

2. In plants, manganese is involved in the light mediated oxidation of H_2O to O_2 in photosystem (II) (Depalmo, 1993).

Applications

1. Manganese is a key component of low cost stainless steel formulations. Manganese is also used to decolourize glass and make violet coloured glass (http://www.lenntech.com/periodic/elements/mn.htm).

2. Manganese can also be added to gold, silver, bismuth etc. to give alloys, which are used for every specific applications generally related to electronic industry. Further, manganese dioxide is used as the cathode (electron acceptor) material in standard and alkaline disposable dry cells and batteries (http://en.wikipedia.org/).

3. Manganese sulphate is used for producing the metal by electrolytic processes in manufacturing inks, varnish, in dyeing and disinfectant (http://nautilus.fis.uc.pt/st2.5/scenes-e/elem/e02530.html).

4. Manganese permanganate is a powerful oxidizing agent used in quantitative analysis technique and in medicine http://www.azom.com/).

Toxicity

1. The symptoms of manganese poisoning are hallucination, forgetfulness, insomnia and lung bronchitis (http://www.lenntech.com /periodic/elements/mn.htm).

2. Manganese toxicity may result in multiple neurological problems. In its worst form manganese toxicity can result in a permanent neurological disorder with symptoms similar to those of Parkinsons' disease, including tremors, difficulty in walking and facial muscle spasm (http://www.chelationtherapyonline.com/).

Conventional remediation methods

High concentration of manganese imparts objectionable and tenacious stains to laundry and plumbing fixtures. Special means such as chemical precipitation, pH adjustment, aeration and use of special ion exchange materials are often necessary for the removal of manganese (Eaton, 1995). Manganese removal by physico-chemical method by aeration and sand filtration can also be used but manganese oxidation kinetics are too slow at pH <9 (http://www.Lenntench.com/processes/iron-manganese/). Manganese is removed by adding some inexpensive basic chemical to the drainage and precipitation of manganese from synthetic solution with sodium hydroxide consistently yields solution free of manganese (http://wvmdtaskforce. com/proceedings/).

Biological method

Nealson (1992) suggested a noble application of manganese oxidizing microorganisms. He noted that manganese oxides with their strong

complexing properties can be used to remove radium from water. Oxidation of Mn^{2+} has been observed by cultures of bacteria, fungi, algae and protozoa (Depalmo, 1993). Manganese oxidation seems to take place inside the cell and by a membrane bound process. The one electron transfer process from Mn^{2+} to Mn^{3+} seems to take place inside the cell plasma. The oxidation of Mn^{3+} to Mn^{4+} appears to be a membrane bound process and the final product precipitate around the cell (Nealson , 2006). Manganese oxide deposits are never found inside cells but always in association with extracellular polymers (Boogerd, 1987). Biogenic manganese oxides can be good tool for soil remediation and heavy metal scavenging. Arsenic as well as metal ions of cadmium, lead, cobalt, mercury and nickel can be adsorbed inexchangeably by biogenic manganese oxides, thus they become immobilized (http://www.up.ethz.ch /education/term_paper/).

Manganese oxidizing bacteria

The manganese oxidizing group is a phylogenetically diverse assemblage, which is characterized by the ability to catalyse the oxidation of divalent soluble Mn^{2+} to insoluble manganese. Manganese oxidizing bacteria are ubiquitous and they can be isolated nearly from any habitat (Nealson, 2006). The genera identified under manganese oxidizing bacteria are *Bacillus, Oceanospirillum, Vibrio, Pseudomonas* and *Leptothrix* (specifically *Leptothrix discophora)* (Gupta et. al, 1987). *Crenothrix* is also manganese oxidizing bacteria form black colour clogging growth in pipelines (Palanichamy et. al, 2002). Pilot scale trickling filter were constructed and tested in order to study biological removal of ammonia, iron and manganese from potable water (Tekerlekopoulou, 2007).

Bacteria that oxidize manganese have a great impact on the redox environment in nature. Bacteria use manganese oxidation to protect themselves from oxidants in their environment. Bacteria can protect themselves with a coating of manganese oxide from predation, viral attacks or heavy metal toxicity. The other advantage of manganese oxidation is the ability of manganese oxides to degrade humic substances oxidatively to smaller compounds. These compounds can be used by the entire microbial community for growth. One possibility is that bacteria use manganese oxidation to derive energy for chemolithautotrophic growth (http://www.up.ethz.ch/education/term_paper/). In this context, manganese oxidations by iron precipitating bacteria were also studied. Different strains of bacteria which oxidise manganese are shown in Figure 17.

Figure 17. Strains of bacteria which oxidise Mn^{2+}

(http://www.up.ethz.ch/education/term_paper/).

Materials and Methods

Screening of isolates

Three isolates obtained from different water and soil samples were studied for manganese oxidation on peptone yeast glucose manganese PYG-Mn medium (Appendix I).

Preparation of 100 ppm manganese sulphate solution

Stock solution was prepared by dissolving 19.82 mg of manganese sulphate in 100 ml sterile distilled water giving 100 ppm manganese sulphate concentration.

Shake flask study of manganese oxidation

Bacillus licheniformis (DI_2), *Enterobacter* sp. (S_4) and *Bacillus cereus* (GP_1) were studied for manganese oxidation. In 250 ml Erlenmeyer flask 90 ml of PYG-Mn broth (Appendix I) were taken containing 10 ml of 100 ppm of manganese sulphate stock solution. The pH of medium was adjusted to 7.4 with 0.1 N NaoH. Actively growing 10% v/v *Bacillus cereus* having 2×10^8 cells/ml was used as inoculum. Uninoculated flask in the experimental sets served as negative control. Flasks were incubated in orbital shaker (Newtronics, India) rotating at 150 rpm at 30±2 ^oC temperature. The sample were collected periodically and titrated with 0.01 N sodium thiosulphate to check manganese oxidation (Appendix II).

Detection of manganese oxidation on solid media

Cultures were streaked on Mn^{2+} containing PYG-Mn agar medium and plates were incubated at 28±2 ^oC temperature. Plates were kept in plastic bags or wrapped with para film to prevent desiccation during incubation. Manganese oxidation was confirmed by benzidine

Results and Discussion

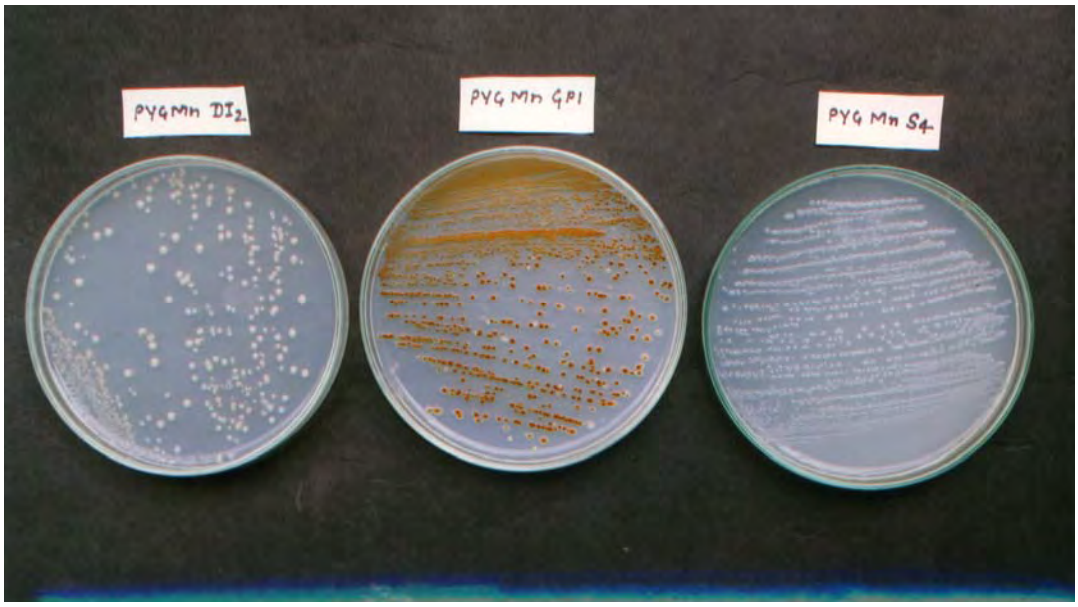
Bacillus licheniformis (DI_2), *Enterobacter* sp. (S_4) and *Bacillus cereus* (GP_1) were studied for manganese oxidation. As shown in Table 34, among the three isolates studied, only *Bacillus cereus* oxidized manganese. It formed dark brown colonies on PYG-Mn agar within 3 to 6 days**.** *Bacillus licheniformis* and *Enterobacter* sp. did not form brown colonies on medium and remained colourless.

Table 34. Screening of isolates for manganese oxidation

Isolates	Manganese oxidation (%)
Bacillus cereus (GP_1)	70
Bacillus licheniformis (DI_2)	-
Enterobacter sp. (S_4)	-

Growth of isolates on PYG-Mn agar medium is depicted in Photograph 16(A). Dark brown coloured colonies of *Bacillus cereus* (GP_1) on PYG-Mn medium is shown in Photograph 16 (B). In PYG-Mn broth *Bacillus cereus* (GP_1) showed 70% of manganese oxidation and results are depicted in Graph 46. *Pseudomonas manganoxidans* and *Arthrobacter globiformis* were reported to form dark brown colonies on PYG-Mn agar. A culture was scored as "manganese oxidizing" only if a visible brown colouration appeared in colonies. Plates were visually observed and the appearance of brown pigment was scored in terms of speed of first appearance of pigment and intensity of colouration after one week. As per literature, Schweisfurth (1973) has examined numerous soil samples, the aquatic and industrial sites containing manganese oxide deposition and isolated about 200 strains of rod shaped bacteria that formed brown colonies on low nutrient Mn^{2+} containing agar (Depalmo, 1993).

(A)

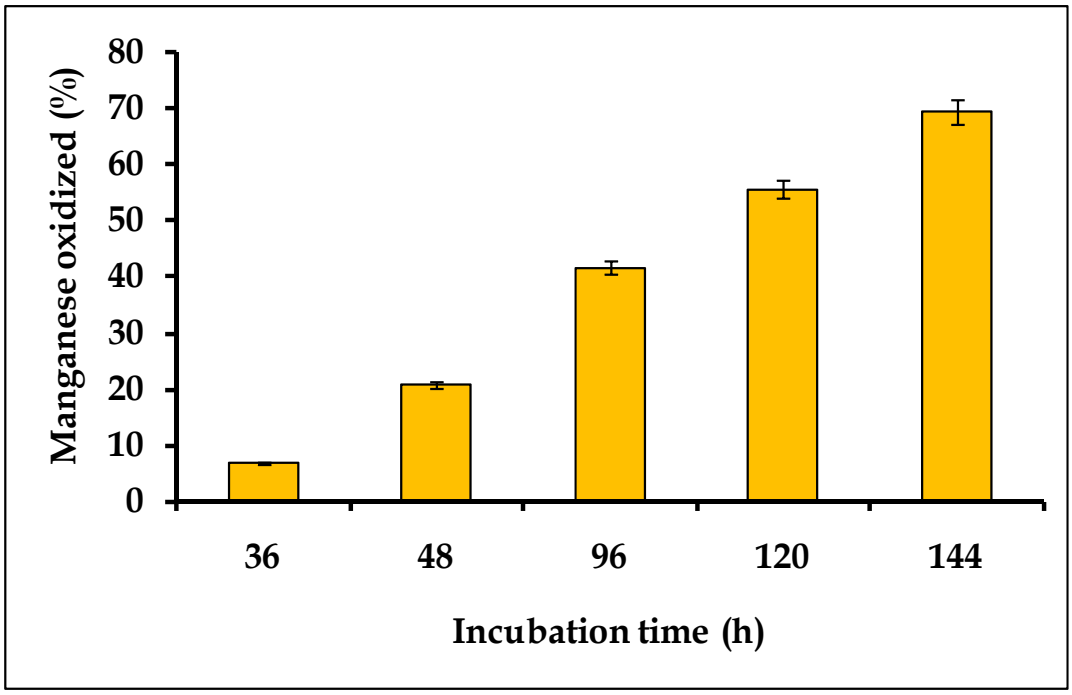


(B)

Photograph 16 (A) Growth of isolates on PYG-Mn agar medium

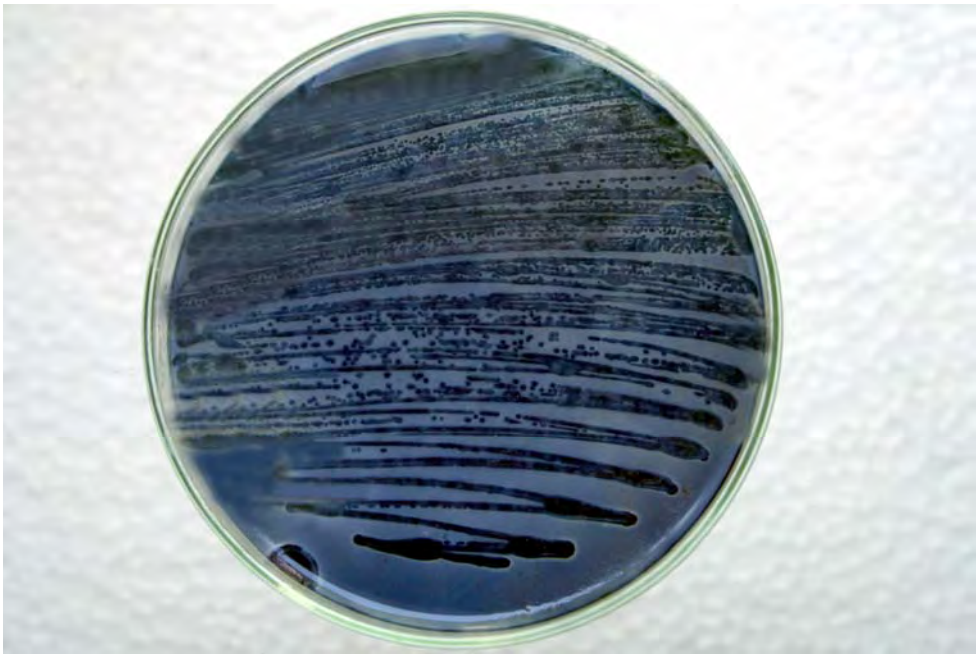
(B) Growth of *Bacillus cereus* (GP_1) on PYG-Mn agar medium.

Graph 46. Manganese oxidation by *Bacillus cereus* (GP_1) in PYG-Mn broth



Confirmatory test of manganese oxidation by benzidine

The manganese oxidizers were recognized by the brown mass of oxidized manganese produced on the colonies and oxidized manganese in such colonies was confirmed by benzidine test (Appendix II) which imparted blue colour to the colonies (Gupta, 1987). The brown colonies developed on plates by *Bacillus cereus* (GP_1) was confirmed by benzidine test, which turned the colonies of oxidizers blue as shown in Photograph 17.



Photograph 17. Confirmatory test of manganese oxidation by benzidine

Conclusions

Significant outcomes from the study undertaken are as follows.

1. Isolation

Isolation of iron precipitating culture from different sources was carried out in citrate agar medium which resulted in isolation of thirty different iron precipitating bacterial cultures. Out of the thirty iron precipitating organisms, ten isolates were selected on the basis of fast visual iron precipitation and their growth on casitone glycerol yeast autolysate (CGY) and nutrient agar medium. Finally three best isolates were selected for further study.

2. Identification

The three iron precipitating cultures were identified as *Enterobacter cloacae* (S_4), *Bacillus licheniformis* (DI_2) and *Bacillus cereus* (GP_1) by 16S rRNA gene sequencing. The sequences of *Enterobacter cloacae* and *Bacillus licheniformis* are deposited in GenBank under the accession no. EU429448 and EU429447.

3. Growth profile study

In tri sodium citrate (TSC) broth and citrate broth *Enterobacter* sp. (S_4) was the fastest growing as compared to *Bacillus cereus (*GP_1) and *Bacillus licheniformis* (DI_2). In tri ammonium citrate (TAC) broth *Bacillus cereus (*GP_1) was fastest growing as compared to *Enterobacter* sp. (S_4) and *Bacillus licheniformis* (DI_2). *Enterobacter* sp. (S_4) gave good growth and iron precipitation in shaking condition as compared to static condition.

4. Metal tolerance study

The *Bacillus cereus (*GP_1*), Bacillus licicheniformis* (DI_2) and *Enterobacte*r sp. (S_4) were found to be resistant up to 80 ppm of copper, chromium, arsenic and cobalt.

5. Iron bioprecipitation study

Enterobacter sp. (S_4) was found to be the most efficient iron precipitating organism among the isolates and removed 96, 94 and 95% iron from 1.0, 0.1 and 0.01 g/L of ferric ammonium citrate in the medium respectively.

6. Copper bioremoval study

Among the studied three isolates, *Enterobacter* sp. (S_4) showed maximum copper removal. So *Enterobacter* sp. (S_4) is a choice of organisms for copper bioremoval study in all other experiments. Copper removal in absence of FAC in medium was very less as compared to presence of FAC (approx 90% removal). This showed that biological activity with FAC play a crucial role in copper remediation. Copper removal increases with increase in pH.

The study was further extended to lab scale column performed at two different modes.

1. Airlifting

2. Without aeration.

- In column study with aeration about 98% and 85% of copper removal was achieved at 50 and 200 ppm of copper in the system. The removal rate varied between 2 to 7 mg/L/h

- In case of absence of aeration, copper removal was 77% and 67% for 50 and 150 ppm copper respectively. The copper removal rate varied between 1.75 to 5 mg/L/h. So, aeration condition proved to be better as compared to absence of aeration.

7. Mercury bioremoval study

Enterobacter sp. (S_4) showed high mercury removal in nutrient broth containing ferric ammonium citrate followed by nutrient broth, citrate broth and marginal removal in minimal medium. As the pH increased, better mercury removal was obtained. For 5, 10 and 15% (v/v) of inoculum size, 80% of mercury removal in nutrient broth containing ferric ammonium citrate was found. In glass column more than 85% of mercury removal was achieved.

8. Cadmium bioremoval study

Enterobacter sp. (S_4) was found to be best for cadmium remediation as compared to B*acillus cereus* (GP_1), *Enterobacter* sp. (S_4) showed higher cadmium removal in citrate broth as compared to nutrient broth containing ferric ammonium citrate and nutrient broth. Cadmium removal was better at pH 5.0 and 7.0. More than 90% of cadmium removal for inoculum size 5, 10 and 15% (v/v) in citrate broth was achieved.

9. Selenite reduction study

In qualitative studies at 50 mM selenite, *Bacillus cereus* (GP_1), *Enterobacter* sp. (S_4) and *Bacillus licheniformis* (DI_2) were found to grow well in casitone glycerol yeast autolysate (CGY) broth. Selenite reduction of approximately 85.2%, 41% and 21% were

observed by these organisms respectively. At 100 mM *Bacillus cereus* and *Enterobacter* sp. (S_4) showed about 64.3% and 32% selenite reduction respectively.

10. Manganese oxidation study

Among the three isolates studied for manganese oxidation, only *Bacillus cereus* (GP_1) formed dark brown colonies on PYG-Mn agar within 4 to 6 days of incubation. *Bacillus licheniformis* (DI_2) and *Enterobacter* sp. (S_4) did not form brown colonies on media and remained colourless. In PYG-Mn broth *Bacillus cereus* (GP_1) showed 70% manganese oxidation.

11. Selected iron precipitating bacterial cultures were successfully exploited for the remediation of heavy metals from aqueous solution at shake flask and column reactors. This indicates the feasibility of these organisms as potential candidates for bioremediation of heavy metals.

Presentations

1. **Rashmi R. Singh, Devayani R.Tipre and Shailesh R.Dave**. Ferrous, manganese oxidation and selenium reduction by *Bacillus* isolate. AMI Conference, 2006, Barkatullah University, Bhopal.
2. **Rashmi R. Singh and Shailesh R.Dave** Bioremediation of copper by *Enterobacter* sp. AMI Conference, 2007, Chennai.
3. **Rashmi R. Singh, Devayani R.Tipre and Shailesh R. Dave.** Bioremediation of Iron by *Enterobacter* sp. National Seminar on New Horizons in Biological Sciences, 2007, Vallabh Vidyanagar.
4. **Rashmi R. Singh, Devayani R. Tipre and Shailesh R. Dave** Bioremediation of mercury by *Enterobacter* sp. AMI 2009, Pune.
5. **Rashmi R. Singh, Devayani R. Tipre and Shailesh R. Dave** Bioremediation of cadmium by *Enterobacter* sp. International Conference on Environmental Issues in Engineering and Advanced Economies: Canada, India, 2009.
6. **Rashmi R. Singh, Devayani R. Tipre and Shailesh R. Dave.** Bioremediation of copper by Environmental isolates.Oral presentation, Vigyan Parishad Science Excellence, 2011, Ahemadabad (2nd Prize).
7. **Rashmi R. Singh, Devayani R. Tipre and Shailesh R. Dave.** Bioremediation of mercury and cadmium by *Enterobacter* sp. Gujarat Science Congress, 2011, oral presentation.

BIOREMEDIATION OF COPPER BY Enterobacter spp.

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INTRODUCTION

- Copper is a malleable metal. Electroplating industrial effluents, mining discharge etc. containing relatively high level of copper released in the environment.
- Concentration of copper beyond 0.05 mg/l is toxic This is one of the biggest threat to human, plants and animals. The most common symptoms of copper toxicity are muscular irritation, hepatic, renal damage and affect nervous system.
- Contamination and high toxicity of copper necessitate their removal from source of pollution of the atmosphere.
- There are physical, chemical and biological methods for heavy metal bioremediation. Microbial cells have potential ability for bioremediation of toxic metals.Microbial species like *Pseudomonas, Bacillus* play significant role in copper removal
- Shake flasks study of metal remediation are reported but column and reactor study data are scanty.

AIMS AND OBJECTIVES

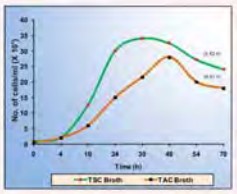
- Isolation of iron precipitating bacteria
- Growth profile of *Enterobacter* spp.in tri-sodium citrate (TSC) and tri-ammonium citrate (TAC) broth was studied.
- Metal tolerance study of *Enterobacter* spp
- Shake flask study was carried out in a range of 10 to 80ppm of copper concentration for copper removal.
- Development of column for copper bioremediation.

MATERIALS AND METHODS

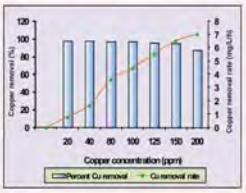
- ISOLATION:
  Enterobacter spp isolated from surface winze water.
- GROWTH PROFILE:
  By spectrophotometric method using tri-sodium and tri-ammonium broth at 540 n/m.
- METAL TOLERANCE STUDY:
  Copper, Chromium, Cobalt and Arsenic in the range of 10 to 80 ppm in citrate broth.
- GLASS COLUMN:
  The supporting material was polystyrene beads. The aerator was connected at the junction of the column and system growth medium was continuously circulated for biofilm formation. Modified media with 20 to 200 ppm copper concentration was passed after biofilm was formed.

AIRLIFT BIOREACTOR

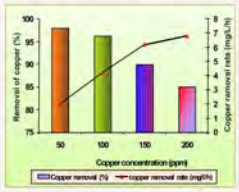
Growth profile of *Enterobacter* spp.



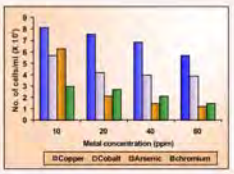
Bioremediation of copper by *Enterobacter* spp.
(50 - 1000 mg/L of nutrients)



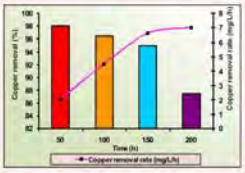
Bioremediation of copper by *Enterobacter* spp.
(0.05 - 1 mg/L of nutrients)



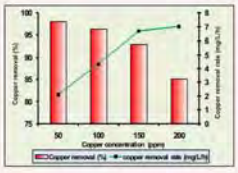
Metal tolerance study of *Enterobacter spp.*



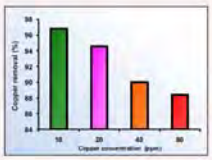
Bioremediation of copper by *Enterobacter* spp.
(1 - 20 mg/L of nutrients)



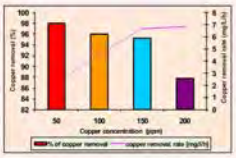
Bioremediation of copper by *Enterobacter* spp.
(0.5 to 10 mg/L of Citrate broth)



Shake flask study of copper removal by *Enterobacter spp.*



Bioremediation of copper by *Enterobacter* spp.
(0.5 - 10 mg/L of nutrients)



CONCLUSIONS

- ✓ Isolate was identified as *Enterobacter* spp.The generation time in TAC broth was more as compared to TSC broth.
- ✓ As metal concentration increases the growth of the *Enterobacter* spp decreases, but it grew even at 80ppm metal concentration. The isolate showed more tolerance for copper and cobalt as compare to arsenic and chromium.
- ✓ In shake flask study at 10, 20, 40 and 80 ppm copper concentration about 97%, 95%, 90% and 88% copper removal was achieved.
- ✓ More than 95% of copper removal was successfully achieved from aqueous solution due to the activity of heterotrophic iron precipitating organisms. The overall remediation rate was found to be 7 mg/l/h for 24 h treatment but it was as high as 40 mg/l/h when it was calculated for first four hour of contact time.
- ✓ So the *Enterbacter spp.* could be exploited for copper bioremediation.

ACKNOWLEDGMENT

We are thankful to Gujarat State Biotechnology Mission (GSBTM), Gandhinagar for the project grant and fellowship to Rashmi Singh.

BIOREMEDIATION OF IRON BY *Enterobacter* spp.

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INTRODUCTION

- Iron is an essential element in human nutrition. It is contained in number of biological significant proteins like Heamoglobin and Ferritin.
- Iron is also an objectionable constituent of drinking water; if its amount exceeds 0.3 ppm concentration the taste and appearance are affected, which has an adverse effect on domestic usage and water supply structures.
- Higher iron concentration stains laundary and plumbing fixtures.
- Excess iron built up in the tissue has been associated with the Heamochromotosis, a disease that causes bronze skin pigmentation, cirrhosis and heart disorders. Iron may cause conjunctivities and retinities. Its contact remain in the tissue.
- Plant grown at higher concentration, shows morphological symptoms of iron toxicity, which causes active oxygen mediated stress in plants.
- Excess Iron in ground water can react with phosphate, which is a nutrient in the form of Tripolyphosphate or with Carbon dioxide, which is produced as a by-product of aerobic respiration. The product of this reaction can adversely affect *In situ* bioremediation system.

AIMS AND OBJECTIVES

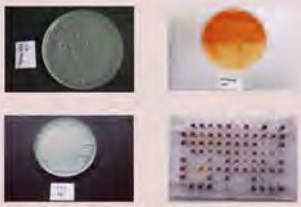
- Isolation of *Enterobacter* spp. from surface winze water.
- Selection of efficient iron precipitating organisms for iron remediation.
- Optimization of iron bioremediation system.

MATERIALS AND METHODS

- Source : Surface Winze Water.
- Media for Isolation : Citrate Agar , Casitone Glycerol Yeast Autolysate and Nutrient Agar.
- Growth pattern and Generation time : Spectrophotometric method using Citrate Broth and Optical density taken at 540 nm.
- Antibiotic Sensitivity Study : Nutrient Agar Medium, using Multidisc PBL, Biodisc 12.
- Metabolic Study : Various biochemical tests viz sugar fermentation , nitrate reduction, MRVP test, phenyl alanine deamination test, urea, starch and gelatin hydrolysis, SIM medium test, oxidase, catalase, triple sugar iron test, Simmons citrate utilization test etc.
- Biolog Study : By using Biolog GN Plate.
- Iron remediation study: Iron precipitation studies were optimized for parameters like inoculum size, pH and at two different concentration 0.1 and 0.01g/L ferric ammonium citrate concentration with inoculum size 50% [v/v].

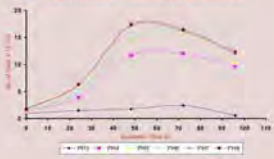
Cellular and colony characteristics of isolate on different media

Characteristic	Citrate Agar	CGY Agar	Nutrient Agar
Cell Morphology			
Size	Medium	Large	Small
Shape	Short rod	Short rod	Short rod
Motility	Motile	Motile	Motile
Arrangement	Cluster	Cluster	Cluster
Grams reaction	Gram negative rod	Gram negative rod	Gram negative rod
Colony Morphology			
Size	Small	Large	Small
Shape	Round	Round	Round
Margin	Entire	Entire	Entire
Elevation	Raised	Raised	Flat
Surface	Smooth	Viscous	Punctiate
Opacity	Opaque	Opaque	Opaque
Appearance	Rust colour mucoid	Creamish white mucoid	White mucoid
Consistency			

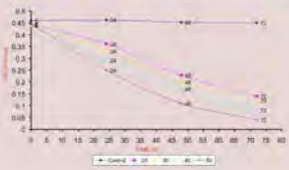


Biolog® plate for culture identification

Growth profile of isolate S4 at various pH



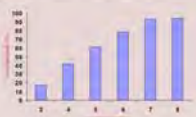
Total iron estimation isolate S4 at different inoculum size



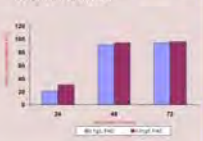
Metabolic characteristics of isolate

Sugar Fermentation			Simmons citrate	+ + +
Glucose	+ + +	+ + +	Starch hydrolysis	+ + +
Sucrose	+ + +	+ + +	Gelatin Hydrolysis	+ +
Lactose	+ +	-	Mucoidway	Large Pinkish colonies, Mucoid, Round, Smooth
Maltose	+ +	+ +	EMB Agar	Opaque Small pink colored Lactose Fermenter Round, Smooth
Mannitol	+ + +	+ +	ITA/KCN	Gram Negative Rod
Fructose	+ + +	+ + +	Oxidase	+
Sorbitol	-	-	Mannolysis	Gram Negative Rod (no slime)
Cellobiose	-	-	H2S	-
Xylose	+ + +	+ + +	Triple Sugar Iron	
NITRATE REDUCTION	+		1. Butt	Acidic
METHYL RED	+		2. Slant	Acidic
VOGES PROSKAUR	-		3. Gas	+
PHENYLALANINE	-		4. H_2S	-
UREA HYDROLYSIS	-		SIM	
OXITIVE FERMENTATION	-		1. Motility	Motile
			2. H_2S Production	-
1. Without oil	+		3. Indole	-
2. With oil	-			

Iron removal at different pH



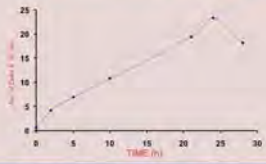
Iron precipitation study with FAC using 50% v/v inoculum size



Antibiogram of S4 isolate

Antibiotics	Antibiotic conc. (µg/disc)	Inhibition zone size(mm)
1.Ampicillin / Sulbactum	20	26
2.Cotrimoxazole	25	20
3.Cefotaxime	30	24
4.Piperacillin	100	Resistant
5.Chloramphenicol	30	28
6.Ciprofloxacin	5	25
7.Ceftizoxime	30	22
8.Tetracycline	30	21
9.Gatofloxcin	10	Resistant
10.Oflaxcin	5	23
11.Gentamicin	10	15
12.Amikacin	30	14

Growth phase of isolate S4 in citrate broth



CONCLUSIONS

- ✓Isolate S4 is a Gram negative straight rod isolated from Surface Winze water which was studied for cellular and colony morphology on various media like Citrate Agar, CGY and Nutrient Agar.
- ✓Generation time and of the isolate in Citrate Broth was found to be 4.21 h. Growth profile was also studied at various pH and growth increases with increasing pH.
- ✓Isolate is sensitive to Chloramphenicol, Ampicillin and Cefotaxime and resistant to Piperacillin and Gatofloxcin among the tested antibiotics.
- ✓Based on metabolic characteristics and Biolog test the isolate was identified as *Enterobacter* spp.
- ✓The isolate showed significant iron precipitating capacity that removed iron 94% and 95.63% at 0.1 and 0.01 g/L of ferric ammonium citrate in the medium respectively. With inoculum size 20, 30, 40 and 50% the iron precipitation was found to be 68.6, 73.98, 79.5 and 93.84%.
- ✓The results showed that large amount of iron could be trapped by *Enterobacter*. So, the *Enterobacter* spp. could be used in iron removal.

ACKNOWLEDGMENT

We are thankful to Gujarat State Biotechnology Mission (GSBTM), Gandhinagar for the project grant and fellowship to Rashmi Singh.

BIOREMEDIATION OF MERCURY BY *Enterobacter* spp.

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INTRODUCTION

- Mercury is given high priority for removal from wastewater because of its high toxicity
- Mercury is released by chloroalkali plants, cement paints, textiles and paper pulp industries.
- The cytotoxicity of mercury is due to the ability to get solubilized into lipids to bind with sulphydryl group of membrane and enzymes to inhibit macromolecular synthesis like transcription and translation.
- The symptoms of mercury toxicity are tremors, gingivitis, renal disorder, nervous failure and also cause Minimata disease and in plants affect photosynthesis. First major incidence of mercury poisoning was reported in 1957. People in Minimata Bay Japan suffered from fatal neurological disease due to the ingestion of mercury loaded fish and shell from the Bay where mercury containing waste were dumped.
- Chang and Hong (1994) reported sorption of mercuric ion by inactivated cells of mercury resistant bacteria *Pseudomonas aeruginosa* and *Bacillus* spp.
- A process based on bioaccumulation of mercury by genetically modified mercury resistant *Pseudomonas putida*, *Aeromonas hydrophila* and natural consortia has been developed in bench scale column.

AIMS AND OBJECTIVES

- To screen the iron precipitating isolate for mercury remediation.
- To study cellular and colony characteristics of the isolate were on different media.
- To study the mercury bioremediation by different organic media containing various concentration of mercury.
- To study the influence of inoculum size and pH on mercury removal in shake flask was done.

MATERIALS AND METHODS

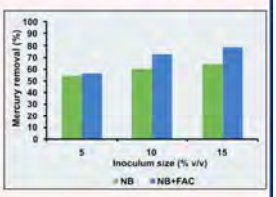
- ISOLATION
  Enterobacter spp. was isolated from winze water, Raichur, Karnataka and *Bacillus* spp. was isolated from gas plant soil sample, Ahmedabad.
- MORPHOLOGY
  Cellular and colony characteristics were studied on three different media viz citrate agar, nutrient agar and casitone yeast autolysate agar.
- MERCURY BIOREMEDIATION STUDY
  Inoculum size (5, 10, 15 % (v/v)), pH and different organic media viz. nutrient broth, citrate agar and nutrient broth containing ferric ammonium citrate were studied for optimization.
- ANALYSIS
  Mercury was estimated by malachite green complex method. Optical density was taken at 630 nm.

GROWTH OF ISOLATE S4 ON NUTRIENT AGAR

CELLULAR AND COLONY CHARACRTERSTICS OF ISOLATE ON DIFFERENT MEDIA

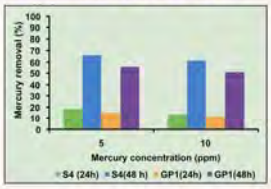
Characteristics	Medium		
	Citrate Agar	CGY Agar	Nutrient Agar
Cell morphology			
Gm	Negative	Long	Small
Shape	Short rods	Short rods	Short rods
Motility	Motile	Motile	Motile
Arrangement	Chains	Chains	Chains
Gram reaction	Gram negative rod	Gram negative rod	Gram negative rod
Colony morphology			
Size	Small	Large	Small
Shape	Round	Irregular	Round
Margin	Entire	Entire	Entire
Elevation	Raised	Raised	Flat
Surface	Smooth	Glazed	Translucent
Opacity	Opaque	Opaque	Opaque
Appearance	Glistening	Glistening	White
Consistency	Viscid	Viscid	Viscid

MERCURY REMOVAL AT DIFFERENT INOCULUM SIZE

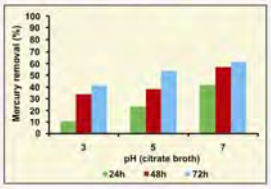


GROWTH OF ISOLATE S4 ON CGY MEDIUM

COMPARISION OF MERCURY REMOVED BY *Bacillus* AND *Enterobacter* AT 5 AND 10 ppm

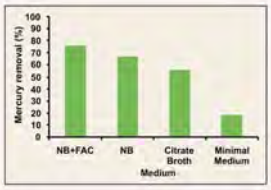


MERCURY REMOVAL AT DIFFERENT pH OF GROWTH MEDIUM



GROWTH OF ISOLATE S4 ON S4 CITRATE AGAR MEDIUM

PERCENT MERCURY REMOVAL IN DIFFERENT ORGANIC MEDIA



AMI 2009, PUNE
15 - 18 December

CONCLUSIONS

- ✓ The isolate S4 (*Enterobacter* spp.) was found to be much more efficient for mercury remediation as compared to Gp1 (*Bacillus cereus*)
- ✓ *Enterobacter sp.* showed higher mercury removal in nutrient broth containing ferric ammonium citrate followed by nutrient broth , citrate broth and very less in minimal media.
- ✓ The percentage of mercury removal was higher in case of NB+ FAC and as compared to NB for inoculum size (v/v) 5,10,15% respectively.
- ✓ Higher the pH better the result of mercury remediation

ACKNOWLEDGMENT

We are thankful to Gujarat State Biotechnology Mission (GSBTM), Gandhinagar for the project grant and fellowship to Rashmi Singh.

BIOREMEDIATION OF CADMIUM BY *Enterobacter* spp.

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INTRODUCTION

- Cadmium being one of the most toxic metals is of particular interest. Cadmium is also carcinogenic and mutagenic. Cadmium is used as plastic stabilizer in manufacturing of nickel cadmium batteries, as colouring pigment product used in fabrics, textiles, paints and in various industrial purposes for electroplating of steel, iron, copper and in pesticides also.
- The most serious effect of cadmium poisoning involves damage to kidney, particularly to renal tube. The symptoms associated with these are proteinurea, glucosurea, bone softening, increase in blood pressure, fatal lung damage and other heart diseases.
- Cadmium uptake by different gram positive organisms like *Staphylococcus*, *Bacillus subtilis* and gram negative bacteria like *Pseudomonas aeruginosa* has been reported.
- Uses of immobilized cells of *Citrobacter* species for effective cadmium removal from liquid industrial waste have been well documented.
- Biosorption of cadmium from solution was studied by using *Streptomyces pimprina* from a Hamycin product plant in Pune. Various species of yeast also have the ability to sequester cadmium from solution.

AIMS AND OBJECTIVES

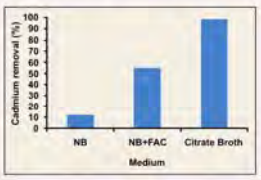
- To screen the iron precipitating isolates for cadmium remediation.
- To study cellular and colony characteristics of the isolate on different media.
- To study the influence of different organic media, inoculum size, pH and ferric ammonium citrate concentration on cadmium removal in shake flask.

MATERIALS AND METHODS

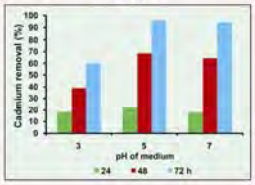
- ISOLATION
  Enterobacter spp. was isolated from winze water and *Bacillus* spp. was isolated from gas plant soil sample.
- MORPHOLOGY
  Cellular and colony characteristics were studied on three different media viz citrate agar, nutrient agar and casitone yeast autolysate agar.
- CADMIUM BIOREMEDIATION STUDY
  Inoculum size (5, 10 and 15%), pH (3, 5 and 7), Ferric ammonium citrate concentration (0.1 and 0.01g/l) and organic media viz nutrient broth, citrate agar and nutrient broth containing ferric ammonium citrate were studied for optimization.
- ANALYSIS
  Cadmium was estimated by Atomic absorption spectrophotometer (Elico Ltd.).

GROWTH OF ISOLATE S4 ON NUTRIENT AGAR

PERCENT CADMIUM REMOVAL IN DIFFERENT ORGANIC MEDIA

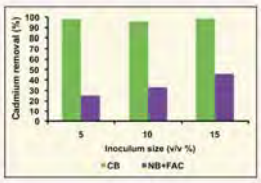


CADMIUM REMOVAL AT DIFFERENT pH OF GROWTH MEDIUM

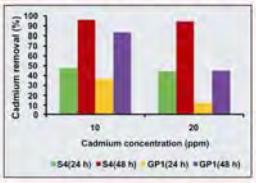


GROWTH OF ISOLATE S4 ON CGY MEDIUM

CADMIUM REMOVAL AT DIFFERENT INOCULUM SIZE

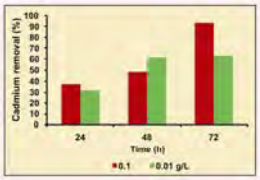


COMPARISION OF CADMIUM REMOVAL BY *Enterobacter* AND *Bacillus* AT 10 AND 20 ppm



GROWTH OF ISOLATE S4 ON S4 CITRATE AGAR MEDIUM

Cadmium removal at different ferric ammonium citrate concentration



CONCLUSIONS

- ✓ The *Enterobacter* spp. was found to be more efficient for cadmium remediation as compared to the Bacillus spp.
- ✓ *Enterobacter* spp. showed higher cadmium removal in citrate broth as compared to nutrient broth and nutrient broth containing ferric ammonium citrate.
- ✓ Cadmium removal was better at pH 5.0 as compared to pH 3.0 and 7.0.
- ✓ The percentage of cadmium removal was higher in case of citrate broth as compared to nutrient broth containing ferric ammonium citrate for inoculum size (v/v) 5, 10 and 15% respectively.
- ✓ Cadmium removal was directly proportional to ferric Ammonium citrate concentration studied.

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6 - 8 December 2009

ACKNOWLEDGMENT

We are thankful to Gujarat State Biotechnology Mission (GSBTM), Gandhinagar for the project grant and fellowship to Rashmi Singh. We are also thankful to faculty members, non-teaching staff and colleagues for the kind support.

GUJARAT SCIENCE CONGRESS 2011 (Oral Presentation)

Bioremoval of mercury and cadmium by *Enterobacter* sp.

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Discharge of industrial, domestic and agricultural wastes in water bodies causes pollution. Such pollutant includes heavy metals, which endanger public health after being incorporated in food chain. Mercury and cadmium are the most toxic pollutants threatening our health and ecosystem. Mercury and cadmium released by various industrial activities such as mining, smelting and electroplating as well as from agriculture such as fertilizer and fungal spray, etc. Higher toxicity necessitates its removal for pollution free environment. The symptoms of mercury toxicity include disruption of nervous system, kidney damage, DNA and chromosomal damage, irritability, restlessness, insomnia, etc. The symptoms of cadmium toxicity include damage to immune system, central nervous system, lung damage, cancer development, high rise of blood pressure and other heart disease. *Enterobacter* sp. plays a significant role in mercury and cadmium bioremoval. Various parameters were studied and optimized for both the metals in shake flask like effect of organic media, influence of inoculum size, effect of pH and effect of ferric ammonium citrate concentration. The *Enterobacter* was identified by 16S rRNA gene sequence, Gene Bank accession no. EU429448. *Enterobacter* shows higher mercury removal in nutrient broth containing ferric ammonium citrate where as higher cadmium removal was achieved in citrate broth. More than 80 % of cadmium and mercury was removed with inoculum size 5, 10 and 15% v/v. Results will be discussed in detail.

VIGYANPARISHAD (2011) (Oral presentation) (2nd prize)

Copper bioremediation by adapted environmental isolates

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Heavy metal pollution is an environmental problem of worldwide concern. The elevated level of copper in the environment has drawn keen attention of environmentalists, because it poses serious threat to mankind as well as flora and fauna. Biosorption of heavy metals by metabolically active and inactive non living biomass of microbial or plant origin is an innovative and alternative for removal of these pollutants from aqueous solution. Hence in the present study, removal of Copper by environmental isolates was studied. Various parameters were optimized such as medium composition, medium pH, concentration of ferric ammonium citrate for maximum copper removal. Among the studied three isolates, *Enterobacter Sp*. Showed maximum copper removal irrespective of copper concentration tested, where as *Bacillus cereus* showed the lowest copper removal among the culture. Both iron and copper precipitation was greatly influenced by the environmental pH. Highest amount of copper was removed at pH 7.0, where almost 84.2% decrease in copper concentration from medium was observed. At pH 5.0 and pH 3.0, 78.3% and 64.5% copper removal was observed. The presence of 1g/L of ferric ammonium citrate showed 2.7 to 4.24 fold increase in copper removal as compared to absence of the salt. The beneficial effect of Ferric ammonium citrate became more prominent as the concentration of copper was increased from 10 to 50 ppm. The result will be discussed in detail.

Appendix I

Media Composition

1. Casitone Glycerol Yeast Autolysate Broth Base (CGY)

Ingredients	g/L
Casitone	5.0
Glycerol	10.0 ml
Yeast Autolysate	1.0
Reagent grade water	1 .0
pH	6.2

2. Nutrient Broth

Ingredients	g/L
Peptone	5.0
Beef extract	3.0
Sodium chloride	5.0
Distilled water	1.0
pH	7.0

In solid medium bacteriological agar was used at a concentration of 30 g/L.

3. Modified Citrate Broth

Ingredients	g/L
Ammonium sulphate	0.5
Sodium nitrate	0.5
Magnesium sulphate	0.5
Dipotassium phosphate	0.5

Calcium chloride	0.2
Ferric ammonium citrate	1.0
Agar	15.0
Final pH	4.5 - 6.6

The medium was solidified by using 2.5% agar and sterilized by autoclaving at 121 °C for 15 minutes.

4. Minimal Medium

Ingredients	**g/L**
Potassium phosphate	15.0
Dipotassium diphosphate	7.0
Ammonium sulphate	1.0
Magnesium sulphate	0.1
pH	7.0

5. PYG-Mn (Peptone Yeast Glucose Manganese Broth)

Ingredients	**g/L**
Bacto peptone	0.25
Bacto yeast extract	0.25
D glucose	0.25
$CaCl_2.2H_2O$	0.070
$MgSO_4.7H_2O$	0.60
Distilled water	1.0
pH	7.0 - 7.4

Glucose was autoclaved separately. For PYG-Mn medium, $MnSO_4$ was added at 10 mg/L concentration.

6. Tri-Sodium Citrate Broth (TSC)

Ingredients	**g/L**
Ammonium sulphate	0.5
Sodium nitrate	0.5
Magnesium sulphate	0.5
Dipotassium phosphate	0.5
Calcium chloride	0.2
Tri- sodium citrate	5.0
Agar	15.0
Final pH	5.5 ± 2.0

7. Tri-Ammonium Citrate (TAC) Broth

Ingredients	**g/L**
Ammonium sulphate	0.5
Sodium nitrate	0.5
Magnesium sulphate	0.5
Dipotassium phosphate	0.5
Calcium chloride	0.2
Tri ammonium citrate	5.0
Agar	15.0
Final pH	5.5 ± 2.0

8. Nutrient Broth+Ferric Ammonium Citrate (NB+FAC)

Ingredients	**g/L**
Peptone	5.0
Beef extract	3.0

Sodium chloride	5.0
Distilled water	1.0
Ferric ammonium citrate	1.0
pH	5.5 ± 6.5

Appendix II

Mercury

1. Preparation of reagents

- 0.001 M standard chloride solution

  Molecular weight of $HgCl_2$ is 271.5 g

  0.1 M $HgCl_2$ = 20.06 mg mercury/ml.

  0.01 M $HgCl_2$=2.006 mg mercury/ml

  0.001 M $HgCl_2$=0.2006 mg mercury/ml

  0.0001 M $HgCl_2$=20 mg mercury/ml

- 0.001 M KI Solution

  Molecular weight of KI is 166.01

  Therefore 0.166 g in 1000 ml distilled water gives 0.001 M KI solution.

- 0.001 M malachite green solution

  Molecular weight of malachite green is 346.5 g

  Therefore 0.346 g in 1000 ml distilled water gives 0.001 M malachite green solution.

- Acetate buffer

  Stock solution

  (A.) 0.2 M solution of acetic acid

  11.55 ml of acetic acid in 1000 ml distilled water

  (B.) 0.2 M solution of sodium acetate

  16.6 g of sodium acetate in 1000 ml distilled water

  Working solution

  2 ml of solution A and 48 ml of solution B are mixed together to give pH 6.0.

- Benzene

  Commercially available AR grade [BDH Chemicals].

Mercury estimation

1. Suitable aliquots of mercuric chloride containing 0 to 20 μg of mercury were taken.
2. 2 ml of KI solution followed by 2 ml of malachite green solution were added.
3. 10 ml of buffer solution was added to adjust the pH of system to 6.
4. After addition of 15 ml of benzene, the mixture was again shaken vigorously for 5 minutes.
5. The extract in the form of organic solvent was collected.
6. The colour intensity of organic layer was measured at 630 nm.
7. A standard curve of optical density versus mercury concentration was plotted.

Copper

Determination of copper as the diethyldithiocarbamate complex

Sodium diethyldithiocarbamate (A) reacts with a slightly acidic or ammonical solution of Cu^{2+} in low concentration to produce a brown colloidal suspension of the cupric diethyldithiocarbamate. The suspension may be extracted with an organic solvent (chloroform, carbon tetrachloride or n-butyl acetate) and the coloured extract determined spectrophotometrically at 560 nm (n butyl acetate) or 435 nm (chloroform or carbon tetrachloride).

Many of the heavy metals give slightly soluble products (some white, some coloured with reagent most of which are soluble in the organic

solvent mentioned. The selectivity of the reagent may be improved by the use of masking agents, particularly EDTA. The reagents decompose rapidly in solution of low pH.

Procedure

1. Dissolve 0.0393 g of A.R. grade cupric sulphate pentahydrate in 1 litre of water in a volume flask.
2. Pipette 10 ml of this solution (containing about 100 μg copper) into beaker.
3. Add 5 ml of 25% aqueous citric acid solution render slightly alkaline with dilute ammonia solution and boil off the excess of ammonia; alternatively adjust to pH 8.5 using pH meter.
4. Add 15 ml of 4% EDTA solution and cool to room temperature.
5. Transfer to a separating funnel; add 10 ml of 0.2% aqueous sodium diethyl dithio carbamate solution. Shake for 45 seconds. A yellow brown colour develops in the solution. Pipette 20 ml of n butyl acetate into the funnel and shake for 30 seconds.
6. The organic layer acquires a yellow colour, cool, shake for 15 seconds and allow phase to separate. Remove the lower aqueous layer; add 20 ml of 5% sulphuric acid (v/v). Shake for 15 seconds, cool and separate the organic phase. Determine the optical density at 560 nm in 1 cm absorption cell against reagent blank. All the copper is removed in one extraction.
7. Repeat the experiment in the presence of 1 mg of Fe^{3+}, no interference can be detected.

Determination of total iron by 1, 10–Phenenthroline spectrophotometric method

Reagents

Stock iron solution

1. Ferrous ammonium sulphate

   Slowly add 20 ml of concentrated H_2SO_4 to 50 ml distilled water and dissolve 1.404 g of ferrous ammonium sulphate Fe $(NH_4)_2(SO_4)_2\,6H_2O$)

   Add 0.1 N potassium permanganate drop wise until a faint pink colour persist. Dilute to 1000 ml and mix

   1 ml = 200 µg Fe

2. Standard iron solution

   Pipette 50 ml stock solution into 1000 ml volumetric flask and dilute to the mark with distilled water.

   1 ml=10 µg Fe

Hydroxylamine solutions

Dissolve 10 g $NH_2OH.HCl$ in 100 ml distill water (stable for month).

Ammonium acetate buffer solution

Dissolve 250g $NH_4C_2H_3O_2$ in 150 ml distilled water. Add 700 ml concentrated (glacial) acetic acid. Final volume will be slightly more than 1000 ml.

1,10 Phenenthroline solution (stable for months)

Dissolve 100 mg of 1,10 phenenthroline monohydrate in 100 ml of water by stirring and heating at 80 °C. Do not boil. Discard the solution, if it darkens. Heating is not required, if two drops of concentrated HCl is added to water (should not be autoclaved).

Procedure

1. Dilute samples that contain iron in the range of 20-200 µg
2. Add distilled water to make volume 33 ml in 50 ml volumetric flask.
3. Add 2 ml concentrated HCl and 1 ml $NH_2OH.HCl$ solution.
4. Add glass beads heat it for 15 minutes.
5. Cool to the room temperature and add 10 ml $NH_4C_3H_2O_2$ buffer solution and 4 ml phenenthroline solution and dilute to mark with distilled water.
6. Mix thoroughly and allow it at least 10-15 minutes for maximum colour development.
7. Measure optical density v/s total iron µg**.**

Selenium Estimation

Reagents

0.1N $KMnO_4$

Dissolve 3.25 g $KmNO_4$ in 700-800 ml distilled water and make volume up to 1000 ml in volumetric flask. Heat the solution to boil and filter the solution by glass wool after cooling it.

0.1 N Ferrous ammonium sulphate

Dissolve 3.9 g ferrous ammonium sulphate in 100 ml distilled water.

Ferroin indicator (0.025 M)

Dissolve 1.485 g orthophenenthroline monohydrate in 100 ml of 0.025 M ferrous sulphate (0.695 g of ferrous sulphate in 100 ml distilled water).

Procedure

1. The selenious acid or selenite corresponding to about 0.1 g of selenium is dissolved in 25 ml of 40% sulphuric acid and diluted to 150 ml.
2. Add 12 g of sodium phosphate or phosphoric acid to prevent formation of Manganese dioxide.
3. Add 50 ml of standard 0.1 N potassium permanganate.
4. Incubate for 30 minutes.
5. Residual potassium permanganate is determined by the addition of slight excess of 0.1 N ferrous ammonium sulphate.
6. Do the back titration with standard 0.1 N potassium permanganate.
7. Add few drops of ferrion indicator for end point approach.
8. Calculation: 1ml = 0.03948 g of selenium

Selenite estimation

Reagents

1. **Selenium standard**

   Dissolve 2.190 g sodium selenite in water in 10 ml HCl and dilute to 1 litre. 1.0 ml = 1.0 mg Se^{4+}
2. **Ammonium Hydroxide** 50% v/v
3. **Cyclohexane**
4. **2,3 Diaminonaphthalene (DAN) solution**

   Dissolve 200 mg DAN in 200 ml 0.1 N HCl, shake for 5 min, extract three times with 25 ml portion at cyclohexane, retain aqueous phase and discard organic portions. Filter (Whatman filterpaper no. 42) into dark containers.

5. **Hydroxyl amine EDTA solution**

   Dissolve 4.5 g Na_2EDTA in 450 ml of water, now add 12.5 g hydroxyl amine hydrochloride adjust volume to 500 ml with distilled water.

   Protocol

   1. Add 2 ml hydroxyl amine EDTA in 10 ml sample.
   2. Adjust to pH 1.5 ± 0.3 with 0.1 N HCl and 50% ammonium hydroxide.
   3. Add 5 ml DAN solution.
   4. Incubate in water bath at 50 °C for 30 min.
   5. Cool and add 4.0 ml cyclohexane.
   6. Cap the container securely and shake vigorously for 5 min.
   7. If the separation is slow, centrifuge for min at 2000 rpm.
   8. Remove aqueous phase.
   9. Take organic phase to capped container.
   10. Take optical density at 480 nm.

Iodometric titration with Sodium thiosulphate for manganese oxidation:

1. Take 10 ml sample from the flask
2. Add 10 ml10% KI in sample
3. Add 15 ml of 2 N HCl and add 3 ml of 1% starch.
4. Titrated with 0.01 N Sodium thiosulphate.

$$Moles\ of\ iodine\ liberated = \frac{Total\ readings\ normality\ of\ sodium\ thiosulphat \times Equivulent\ weight\ of\ iodine}{1000 \times Molecular\ weight\ of\ iodine}$$

Confirmatory test by Benzidine

Take 200 mg of Benzidine in 1 ml of glacial acetic acid and 10 ml of ethanol. Pour 4-5 ml solution on PYGMn agar plate, the manganese oxidized colonies turned blue.

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