Biosorption of Heavy Metals using Individual and Mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis*

Thesis submitted

by

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CERTIFICATE

This is to certify that the project report titled "**Biosorption of heavy metals using individual and mixed cultures of *Bacillus subtilis* and *Pseudomonas aeruginosa***". has been done under my guidance is a bonafide record of work done by **Ms. K. Tarangini** in partial fulfillment of the requirement for the completion of the Master of Technology in Chemical Engineering.

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Abstract

Biosorption can be an effective technique for the treatment of heavy metal bearing waste water resulting from humuns and industrial activities. Several gram positive and gram negative bacteria have the ability to remove the heavy metals and there by making water contaminant free. It has been reported that attenuated bacterial biomass have greater biosorption capability than viable cells. In the present study, the biosorption of heavy metals using individual and mixed culture of attenuated bacteria (gram positive and gram negative) like *Bacillus subtilis* and *Pseudomonas aeruginosa* and parameters affecting the biosorption of heavy metals; such as time, pH, biomass concentration and initial metal concentration have been investigated. The batch experiments have been carried out using individual and mixed bacterial culture and the biosorption parameters were optimized using univariate procedures. The present study shows that 90.4% of biosorption of Mercury was observed for mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* and 99.3% and 78.5% biosorption for individual cultures respectively. The time taken for maximum sorption of Mercury was 60, 40 and 40 minutes for mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis.* The optimum biomass concentration was found to be 2, 0.5 and 2.5 mg/ml for mixed cultures, *Pseudomonas aeruginosa* and *Bacillus subtilis*. pH 5 was found to be optimum for all the three biomass (two individual cultures; one mixed culture) for Mercury biosorption. Optimum temperature was 32^{o}C for all the three systems used in the present work. Adsorption isotherms of all the three metals with mixed cultures were best fitted with Langmuir and Freundlich isotherm models having highest value of regression coefficients with R^2 0.99 which is close to one. Two kinetic models namely pseudo first order equation and pseudo second order equation were also tested for the biosorption processes. The biosorption of Chromium (VI) shows that 77.6% for mixed cultures, 60.5 and 81.3 for *Pseudomonas aeruginosa* and *Bacillus subtilis* respectively. The optimum biomass concentration was found to be 1.5, 1.5 and 2 for mixed cultures (*Pseudomonas aeruginosa* and *Bacillus subtilis)* at 32^{o}C and 3 pH. The equilibrium Arsenic biosorption were also conducted by using the same biomasses as mentioned above by achieving a sorption of 30%, 32% and 28% for mixed culture, (*Pseudomonas aeruginosa* and *Bacillus subtils).* pH 5 was found to be optimum for mixed cultures (*Pseudomonas aeruginosa* and *Bacillus subtils)* and

Pseudomonas aeruginosa cultures and pH 6 for *Bacillus subtilis* cultures. 32^{o}C temperature was found to be optimum for the biosorption process of Arsenic. The equilibrium time was 15min for *Pseudomonas aeruginosa* and mixed culture, and 20 min for *Bacillus subtilis* for Arsenic. Experiments were also conducted for binary system of heavy metal in aqueous solutions using the mixed culture. The binary aqueous solutions of Mercury-Chromium (VI) and Mercury-Arsenic were also used in the present work, where a maximum sorption of 74% and 30% for Chromium (VI) and Mercury was observed respectively, and the optimum condition of pH-4, temperature 32^{o}C and 2mg/ml biomass concentration. In Mercury-Arsenic binary aqueous solution, the removal of 70.7% for Mercury and 20.9% for Arsenic at pH-5, temperature 32^{o}C and biomass concentration of 3mg/ml were also reported in the present work.

Chapter 1

1. Introduction

Earth's surface comprises of 70% water is the most valuable natural resource existing on our planet. Without this invaluable compound, the life on the Earth would not exist. Although this fact is widely recognized, pollution of water resources is a common problem being faced today. Heavy metal pollution occurs directly by effluent outfalls from industries, refineries and waste treatment plants and indirectly by the contaminants that enter the water supply from soils/ground water systems and from the atmosphere via rain water. (Vijayaraghavan and Yun, 2008) Modern industry is, to a large degree, responsible for contamination of the environment. Lakes, rivers and oceans are being overwhelmed with many toxic contaminants. Among toxic substances reaching hazardous levels are heavy metals. (Vieira and Volesky, 2000) Heavy metals are the group of contaminants of concern, which comes under the inorganic division. Some strong toxic metal ions such as Hg are very toxic even in lower concentration of 0.001-0.1 mg/ L. Metals are extensively used in several industries, including mining, metallurgical, electronic, electroplating and metal finishing. The presence of metal ions in final industrial effluents is extremely undesirable, as they are toxic to both lower and higher organisms. Under certain environmental conditions, metals may accumulate to toxic levels and cause ecological damage (Jefferies and Firestone, 1984). Of the important metals, Mercury, lead, cadmium, Arsenic and Chromium (VI) are regarded as toxic; whereas, others, such as copper, nickel, cobalt and zinc are not as toxic, but their extensive usage and increasing levels in the environment are of serious concerns (Brown and Absanullah, 1971; Moore, 1990; Volesky, 1990). Various techniques have been employed for the treatment of metal bearing industrial effluents, which usually include precipitation, adsorption, ion exchange, membrane and electrochemical technologies but these techniques are expensive, not environment friendly and usually dependent on the concentration of the waste which are ineffective in very diluted solutions. Therefore, the

search for efficient, eco-friendly and cost effective remedies for wastewater treatment has been initiated. It was only in the 1990s that a new scientific area developed that could help to recover heavy metals and it was bioremediation. The early reports described how abundant biological materials could be used to remove, at very low cost, even small amounts of toxic heavy metals from industrial effluents. The principle advantages of biological technologies for the removal of pollutants are they can be carried out in situ at the contaminated site, usually environmentally benign (no secondary pollution) and they are cost effective. Of the different biological methods, bioaccumulation and biosorption have been demonstrated to possess good potential to replace conventional methods for the removal of metals (Volesky and Holan, 1995; Malik, 2004).

Some confusion has prevailed in the literature regarding the use of the terms "bioaccumulation" and "biosorption" based on the state of the biomass. Herein, therefore, bioaccumulation is defined as the phenomenon of living cells; whereas, biosorption mechanisms are based on the use of dead biomass. To be precise, bioaccumulation can be defined as the uptake of toxicants by living cells. The toxicant can transport into the cell, accumulate intracellularly, across the cell membrane and through the cell metabolic cycle (Malik, 2004). Conversely, biosorption can be defined as the passive uptake of toxicants by dead/inactive biological materials or by materials. Metal-sequestering properties of non-viable biomass provide a basis for a new approach to remove heavy metals when they occur at low concentrations (Volesky, 1990). That aspect of biosorption makes the eventual recovery of this waste metal easier and economical. This study aimed to investigate the potential of mixed cultures of gram positive and gram negative bacteria *Bacillus subtilis* and *Pseudomonas aeruginosa*. A comparative work was done on single cultures of both the bacteria. Here the metals studied are Arsenic, Chromium and Mercury. The parameters are optimized for all the there metal sorption studies. Studies were performed for the binary solutions of Mercury-Arsenic and Mercury- Chromium.

1.1 Mercury

Mercury is a naturally occurring metallic element that is found in soil, air, and water. Mercury is present in many forms such as elemental or metallic Mercury, inorganic Mercury compounds, and organic Mercury compounds. Mercury combines

with elements, such as chlorine, sulfur, or oxygen to form inorganic Mercury compounds or may combine with alkyl and aryl organic groups to form organic Mercury compounds.

1.1.1 Uses of Mercury

Being the only metal which is liquid at room temperature Mercury has some specialist uses:

Table-1.1: Significant uses of Mercury

Thermometers	Barometers
Manometers	Diffusion pumps and other instruments
Light switches	Paints
Making batteries (Mercury cells)	Mercury-vapour lamps and advertising signs
Dental amalgams	Caustic soda and chlorine production
Pesticides	Antifouling paints

1.1.2 Sources of Mercury pollution

Based on our present level of understanding, the *Mercury Report to Congress* (USEPA, 1997b) suggested that the flux of Mercury from the atmosphere to a location on the earth's surface was comprised of contributions from:

- The natural global cycle;
- The global cycle perturbed by human activities;
- Regional sources; and
- Local sources.

Volcanic activity, geothermal activity, and natural cinnabar deposits are obvious natural sources. Transport and conversion of the Mercury to different forms from these sources is also natural and can occur anywhere on the planet. Mercury can enter the environment from a variety of anthropogenic or manmade sources. These sources include the following:

- Combustion of coal in coalfired power plants,
- Municipal waste incinerators, hospitals and crematoriums, dental offices,
- Thermal treatment of gold and Mercury ores,
- Releases of Mercury from current and legacy mining operations, and

- Geothermal heat recovery processes,
- Munitions Operations

Lesser known generators of Mercury in the environment include crematoriums, dental, offices and munitions operations. (Mercury source protocol, 2008)

1.1.3 Mercury Health Issues and Toxicity

Mercury is a highly toxic heavy metal. Even at low levels, Mercury can affect the central nervous system and in particular, the brain. At higher levels of Mercury, other organs, such as the kidneys, are susceptible to damage. Inorganic Mercury, present in water sediments, is subject to bacterial conversion to methyl Mercury compounds that are bioaccumulated in the aquatic food chain to reach the highest concentration in predatory fish. Human exposure to Mercury vapor is from dental amalgam and industries using Mercury. Methyl Mercury compounds is found exclusively in seafood and freshwater fish. The health effects of Mercury vapor have been known since ancient times. Severe exposure results in a triad of symptoms, erethism, tremor, and gingivitis. Subtle effects such as preclinical changes in kidney function and behavioral and cognitive changes associated with effects on the central nervous system. Methyl Mercury is a neurological poison affecting primarily brain tissue. In adults, brain damage is focal affecting the function of such areas as the cerebellum (ataxia) and the visual cortex (constricted visual fields). Methyl Mercury also at high doses can cause severe damage to the developing brain. Today the chief concern is with the more subtle effects arising from prenatal exposure such as delayed development and cognitive changes in children. Studies have shown that children and developing fetuses are at a higher risk for developing problems when exposed to Mercury. Methyl Mercury and metallic vapors are the most harmful forms of Mercury in that these forms easily reach the brain (ATSDR, 1999). Mercury toxicity also impacts reproduction in fish species. Mercury can impact aquatic plants in a variety of ways across a wide range of concentrations, including survival and growth. These impacts are partially related to disruption of the photosynthesis process.

Cutaneous Diseases Caused by Mercury (http://www.drMercury.com/library.html)

1. Grovers's disease (transient acantholytic dermitis)
2. Generalized and localized popular eruption
3. Pustolosis palmaris et plataris

4. Persistant palmar or plantar plaques
5. Atypical eczemas
6. Guttate psoriasis and atopic dermatitis.

Systemic Symptoms and Signs Which Help to Diagnosis Mercury Toxicity

1. Difficulty in sleeping
2. Difficulty in memory and concentration
3. Depression
4. Tiredness
5. Tremor (usually seen in blood levels greater then 10 micro grams/liter)
6. Irritability
7. Dizziness or vertigo
8. Rectal bleeding, including colon cancer in patients over 50
9. Idiopathic atrial fibrillation or other cardiac arrhythmia
10. Pain or numbness in arms and legs including plantar fasciitis
11. Diagnosis of muscular degeneration, Parkinson's disease or essential tremor or Alzheimer's disease.
12. Infertility
13. Recurrent alopecia areata (http://www.drMercury.com/library.html)

1.1.4 Permissible limits of Mercury

Maximum Contaminant Level inorganic Mercury in drinking water = 0.002 mg/L (USEPA 2003), Permissible upper limit for Hg in foods should be 0.05 parts per million, (Goldwater, 1971).

1.2 Chromium

Due to increase in population coupled with mining, extraction and use if various metals as different industrial and household materials, the load of toxic metal pollution in the environment are increasing. Vauquelin discovered the existence of Chromium in 1789 (James et al., 1997). Cr (III) occurs naturally in soils and mineral deposits, while Cr (VI) is a product of man's activity and is rarely encountered in natural, unpolluted soils.

1.2.1 Uses of Chromium

Chromium chemicals are widely distributed and used in both developing and industrialized nations for myriad industrial and commercial products (Table-5.1).

Table-1.2: Uses of Chromium.

Wood preservative	Oxiding agent
Metal finishing	Catalysis
Leather tanning	Ceramic coatings
Pigments	Abrasives and refractories
Textile mordant	Safety matches
Magnetic tape	Glues and adhesives
Colored glass	Etchant for plastics

1.2.2 Sources of Chromium pollution

Chromate is ranked among the top 20 contaminants of concern in the world. Chromium in natural solids varies widely with the type and nature of the rock or sediment deposit. Among different natural solids, shales, lithosphere, sandstone, and river suspended matter typically exlubit relatively high concentrations of Chromium, while carbonates, granite, and sandy sediments generally contain low concentrations of Chromium. When found in geologic deposits, Chromium is principally identified as chromites (Fe0.Crz03).

- Naturally occurring Chromium concentrations in water arise from mineral weathering process, soluble organic Chromium, sediment load and precipitation. However, such concentrations in natural water bodies are very low, in the order of 10pg per ml or so.
- Major content of Chromium in water bodies comes from industrial and domestic wastewater. Industrial sources are known contribute 68% of Chromium in the influent to sewers.
- Chromium containing effluents are released by following activities: metal plating, anodizing, ink manufacture, dyes, pigments, glass, ceramics, glues, tanning, wood preserving, textiles and corrosion inhibitors in cooling water. Both Cr (111) and Cr (VI) can be present in these effluents.

1.2.3 Chromium Health Issues and Toxicity

There are three different routes of entry for Chromium into the human body. The gastro- intestinal route is the most important physiological condition, while in occupational exposure the Etchant for plastics airways are more important routes of entry and uptake. The va!ency state of Chromium, water solubility, acidity of gastric juice and the passage time through the tract are the factors which control the uptake of Chromium in the gastro-intestinal tract, while uptake in the airways is influenced by the particle size distribution and also on factors which govern the clearance time from the lungs. The third route is through the epidermis and this is very significant in pathological conditions.

Trivalent Chromium has low acute and chronic toxicity to humans at high doses, however in lower concentration, it is considered as an essential trace nutrient. Cr (III) deficiency is characterized by impaired growth and longevity. There is also evidence that Cr (III) is involved in the glucose tolerance of the man. The inability of Cr (III) to penetrate cell membranes severely limits or precludes the possibility of carcinogenic activity.

The Cr (VI) form however is toxic even in small amounts. Cr (VI) diffuses through the epidermis and is readily reduced to Cr (III) by gastric fluids, extra-cellular and intra-cellular low molecular weight molecules and proteins. The Cr (III) thus formed interacts with nuclear enzymes, proteins nucleotide and DNA. This constitutes for the mutagenic and carcinogenic activity of Cr (VI) (Snow, 1994) Thus the reduction of Cr (VI) to Cr (III) and its subsequent removal is of great importance to mankind.

1.2.4 Permissible limits of Chromium

According to the Indian standars (Baral, 2006), the permissible limit of Cr (VI) is 0.05 and 0.1 mg/L for potable and industrial discharge water respectively.

Biosorption studies of Chromium were done using individual and mixed cultures of *Bacillus* and *Pseudomonas* species (1:1). Under optimized conditions a sorption of 81% and 60% for individual cultures and 78% for mixed cultures was obtained. Biosorption studies on binary solutions of Chromium and Mercury are performed the results are discussed in the thesis.

1.3 Arsenic

Arsenic is the main constituent of more than 200 mineral species, of which about 60% are arsenate, 20% sulfide and sulfosalts and the remaining 20% include arsenides, arsenites, oxides and elemental Arsenic (Onishi, 1969). The most common of the Arsenic minerals is arsenopyrite, (FeAsS,). It was estimated that about one-third of the atmospheric flux of Arsenic is of natural origin.

1.3.1 Sources and occurrence of Arsenic in the environment

Volcanic action followed by low-temperature volatilization is the most important natural source of Arsenic. Inorganic Arsenic of geological origin is found in groundwater used as drinking-water in several parts of the world, for example Bangladesh. Organic Arsenic compounds such as arsenobetaine, arsenocholine, tetramethylarsonium salts, arsenosugars and Arsenic containing lipids are mainly found in marine organisms although some of these compounds have also been found in terrestrial species. Arsenic is found associated with many types of mineral deposits, especially those including sulfide mineralization (Boyle & Jonasson, 1973). Elemental Arsenic is produced by reduction of Arsenic trioxide (As_2O_3) with charcoal. As_2O_3 is produced as a by-product of metal smelting operations. It has been estimated that 70% of the world Arsenic production is used in timber treatment as copper chrome arsenate (CCA), 22% in agricultural chemicals, and the remainder in glass, pharmaceuticals and non-ferrous alloys. Mining, smelting of non-ferrous metals and burning of fossil fuels are the major industrial processes that contribute to anthropogenic Arsenic contamination of air, water and soil. Historically, use of Arsenic-containing pesticides has left large tracts of agricultural land contaminated. In 1983, Arsenical pesticides were one of the largest classes of biocontrol agent in the USA (Woolson, 1983). From the 1960s there was a shift, in herbicide use, from inorganic compounds (including lead and calcium arsenate and copper acetoarsenite) to inorganic and organic compounds. The use of Arsenic in the preservation of timber has also led to contamination of the environment.

1.3.2 Effects on human health

Soluble inorganic Arsenic is acutely toxic, and ingestion of large doses leads to gastrointestinal symptoms, disturbances of cardiovascular and nervous system functions, and eventually death. In survivors, bone marrow depression, haemolysis, hepatomegaly,

melanosis, polyneuropathy and encephalopathy may be observed. Long-term exposure to Arsenic in drinking-water is causally related to increased risks of cancer in the skin, lungs, bladder and kidney, as well as other skin changes such as hyperkeratosis and pigmentation changes. Increased risks of lung and bladder cancer and of Arsenic-associated skin lesions have been reported to be associated with ingestion of drinking-water at concentrations ≤50μg Arsenic/litre. Occupational exposure to Arsenic, primarily by inhalation, is causally associated with lung cancer. Increased risks have been observed at cumulative exposure levels ≥0.75 (mg/m3) year (e.g. 15 years of exposure to a workroom air concentration of 50μg/m3). However, there is good evidence from studies in several countries that Arsenic exposure causes other forms of PVD. Conclusions on the causality of the relationship between Arsenic exposure and other health effects are less clear-cut. The evidence is strongest for hypertension and cardiovascular disease, suggestive for diabetes and reproductive effects and weak for cerebrovascular disease, long-term neurological effects, and cancer at sites other than lung, bladder, kidney and skin. Arsenic contamination leads to lethality, inhibition of growth, photosynthesis and reproduction, and behavioral effects.

1.3.3 Permissible limits of Arsenic

The level of Arsenic allowed in drinking water has been set at 0.01 mg/l by the World Health Organization (WHO). No samples of rice grain (*Oryza sativa* L.) had Arsenic concentrations more than the recommended limit of 1.0 mg/kg. According to FAO/WHO report, the value is 2.1 μg/kg body wt./day). According to WHO, intake of 1.0 mg of inorganic Arsenic per day may give rise to skin lesions within a few years. Arsenic in groundwater above the WHO maximum permissible limit of 0.05 mg l^{-1} has been found in six districts of West Bengal.

Biosorption studies of Arsenic were done using individual and mixed cultures of *Bacillus* and *Pseudomonas* species (1:1). Under optimized conditions a sorption of 28% and 32% for individual cultures and a sorption of 30% were observed. Biosorption studies on binary solutions of Arsenic and Mercury were performed whose optimum parameters are discussed in the thesis.

Chapter 2

2. Literature survey

2.01 Heavy metal

Heavy metals are defined as metals with a specific weight usually more than 5.0 g/cm^3, which is five times higher than water. The toxicity of heavy metals occurs even in low concentrations of about 1.0-10 mg/L. Of the 90 naturally occurring elements, 21 are non-metals, 16 are light-metals and the remaining 53 (with As included) are heavy-metals. Most heavy metals are transition elements with incompletely filled d orbitals. These d orbitals provide heavy-metal cations with the ability to form complex compounds which may or may not be redox-active. Thus, heavy-metal cations play an important role as trace elements in sophisticated biochemical reactions (Nies, 1999). A trace element is considered essential if it meets the following criteria: it is present in all healthy tissues of living things; its concentration from one animal to the next animal is fairly constant; its withdrawal from the body induces, reproducibly the same physiological and structural abnormalities regardless of the species studied; its addition either reverses or prevents these abnormalities; the abnormalities induced by deficiency are always accompanied by pertinent, significant biochemical changes and these biochemical changes can be prevented or cured when the deficiency is corrected. A total of 30 elements are now believed to be essential to life. They can be divided into the 6 structural elements, 5 macro minerals and 19 trace elements (Florence, 1989). Virtually, all metals whether essential or inessential can exhibit toxicity above certain threshold concentrations which for highly toxic metal species may be extremely low. The toxicity caused by heavy-metals is generally a result of strong coordinating abilities (Gadd, 1992). Certain metals have been known to be toxic for centuries. For example, Theophrastus of Erebus (370-287 B.C.) and Pliny the Elder (23-79) both described poisonings that resulted from Arsenic and Mercury. Other heavy-metals, such as cadmium were not recognized as poisonous until the early nineteenth century (Young, 2000).

Based on the physiological effect and toxicity, heavy metals are classified as follows

Table-2.1: Classification of heavy metals based on toxicity (Thakur, 2006).

Fe, Mo, Mn	Low toxicity
Zn, Ni, Cu, V, Co, W, Cr	Average toxicity
As, Ag, Sb, Cd, Hg, Pb, U	High toxicity

2.02 Biogeochemistry of Heavy-metals

Heavy-metals occur naturally in the environment in rocks and ores and cycle through the environment by geological and biological means. The geological cycle begins when water slowly wears away rocks and dissolves the heavy-metals. The heavy-metals are carried into streams, rivers, lakes and oceans and may be deposited in sediments at the bottom of the water body or they may evaporate and be carried elsewhere as rainwater. The biological cycle includes accumulation in plants and animals and entry into the food web (Young, 2000). Some heavy-metals are not available to the living cell in the usual ecosystems. They may be present in the earth's crust only in very low amounts or the ion of the particular heavy-metal may not be soluble (Nies, 1999).

2.03 Heavy metal contamination and Toxicity

Heavy Metal Contamination is a general term given to describe a condition having abnormally high levels of toxic metals in the environment. Heavy metals are subtle, silent, stalking killers. It has been realized that sometimes the natural cycles can pose a hazard to human health because the level of heavy-metals exceed the body's ability to cope with them. The situation becomes worst by the addition of heavy-metals to the environment as a result of both the rapidly expanding industrial and domestic activities. The metals are introduced into the environment during mining, refining of ores, combustion of fossil fuels, industrial processes and the disposal of industrial and domestic wastes (Xie et al., 1996). Human activities also create situations in which the heavy-metals are incorporated into new compounds and may be spread worldwide (Young, 2000). Many aquatic environments face metal concentrations that exceed water

criteria designed to protect the environment, animals and humans. Every essential element is toxic if taken in excess and there is a safe window for essential dose between deficiency and toxicity. Some elements such as Ca and Mg have wide window whereas others such as Se and F have narrow window where by an excess will rapidly lead to toxicity and death. Metal toxicity can be divided into three categories i.e. blocking the essential biological functional groups of molecules, displacing the essential metal ion in biomolecules and modifying the active conformation of biomolecules (Florence, 1989). The toxicity effects greatly depend on the bioavailability of the toxicant meaning the proportion of the contaminant present in the environment in the form(s) that can be assimilated by organism (Petänen, 2001). The health hazards presented by heavy-metals depend on the level of exposure and the length of exposure. In general, exposures are divided into two classes: acute exposure and chronic exposure. Acute exposure refers to contact with a large amount of the heavy-metal in a short period of time. In some cases the health effects are immediately apparent; in others the effects are delayed. Chronic exposure refers to contact with low levels of heavy-metal over a long period of time (Young, 2000).

2.04 Conventional methods of metal ion removal and disadvantages

Many procedures have been applied in order to remove heavy-metals from aqueous streams. Among the most commonly used techniques are chemical precipitation, chemical oxidation and reduction, ion-exchange, filtration, electrochemical treatment, reverse osmosis (membrane technologies), evaporative recovery and solvent extraction (Xia and Liyuan, 2002). These classical or conventional techniques give rise to several problems such as unpredictable metal ions removal and generation of toxic sludge which are often difficult to dewater and require extreme caution in their disposal (Xia and Liyuan, 2002). Besides that, most of these methods also present some limitations whereby they are only economically viable at high or moderate concentrations of metals but not at low concentrations (Addour et al., 1999), meaning diluted solutions containing from 1 to 100 mg/L of dissolved metal(s) (Cossich et al., 2002). Heavy metal removal by classical techniques involves expensive methodologies. These are due to high energy and

reagent requirements (Xia and Liyuan, 2002). Some of them are explained in brief with their disadvantages.

2.04.1 Reverse Osmosis

It is a process in which heavy metals are separated by a semi-permeable membrane at a pressure greater than osmotic pressure caused by the dissolved solids in wastewater. The disadvantage of this method is that it is expensive.

2.04.2 Electro dialysis

In this process, the ionic components (heavy metals) are separated through the use of semi-permeable ion selective membranes. Application of an electrical potential between the two electrodes causes a migration of cations and anions towards respective electrodes. Because of the alternate spacing of cation and anion permeable membranes, cells of concentrated and dilute salts are formed. The disadvantage is the formation of metal hydroxides, which clog the membrane. The disadvantage is the formation of metal hydroxides, which clog the membrane.

2.04.3 Ultra filtration

They are pressure driven membrane operations that use porous membranes for the removal of heavy metals. The main disadvantage of this process is the generation of sludge.

2.04.4 Ion-exchange

In this process, metal ions from dilute solutions are exchanged with ions held by electrostatic forces on the exchange resin. The disadvantages include high cost and partial removal of certain ions.

2.04.5 Chemical Precipitation

Precipitation of metals is achieved by the addition of coagulants such as alum, lime, iron salts and other organic polymers. The large amount of sludge containing toxic compounds produced during the process is the main disadvantage. (Ahalya et al., 2003)

The above techniques can be summarized as expensive, not environment friendly and usually dependent on the concentration of the waste. Therefore, the search for efficient, eco-friendly and cost effective remedies for wastewater treatment has been initiated. In recent years, research attention has been focused on biological methods for the treatment of effluents, some of which are in the process of commercialization (Prasad

and Freitas, 2003). There are three principle advantages of biological technologies for the removal of pollutants; first, biological processes can be carried out in situ at the contaminated site; Second, bioprocess technologies are usually environmentally benign (no secondary pollution) and third, they are cost effective. Of the different biological methods, bioaccumulation and biosorption have been demonstrated to possess good potential to replace conventional methods for the removal of metals. (Volesky and Holan, 1995; Malik, 2004).

2.05 Bioremediation

Bioremediation can be defined as any process that uses microorganisms, fungi, green plants or their enzymes to return the natural environment altered by contaminants to its original condition. Heavy metal bioremediation involves removal of heavy metals from waste water and soil through metabolically mediated or physico-chemical pathways. Algae, bacteria and fungi and yeasts have proved to be potential in metal removal from the waste waters (Volesky, 1986).

2.05.1 Advantages of Bioremediation

- Low cost
- High efficiency
- Minimization of chemical and biological sludge
- Regeneration of biosorbents and
- Possibility of metal recovery.

2.06 Mechanisms involved in Bioremediation

The complex structure of microorganisms implies that there are many ways for the metal to be taken up by the microbial cell. The bioremediation mechanisms are various and are not fully understood. They may be classified according to various criteria.

- According to the dependence on the cell's metabolism
  - Metabolism dependent and
  - Non -metabolism dependent
- According to the location where the metal removed from solution is found
  - Extra cellular accumulation/ precipitation
  - Cell surface sorption/ precipitation and
  - Intracellular accumulation

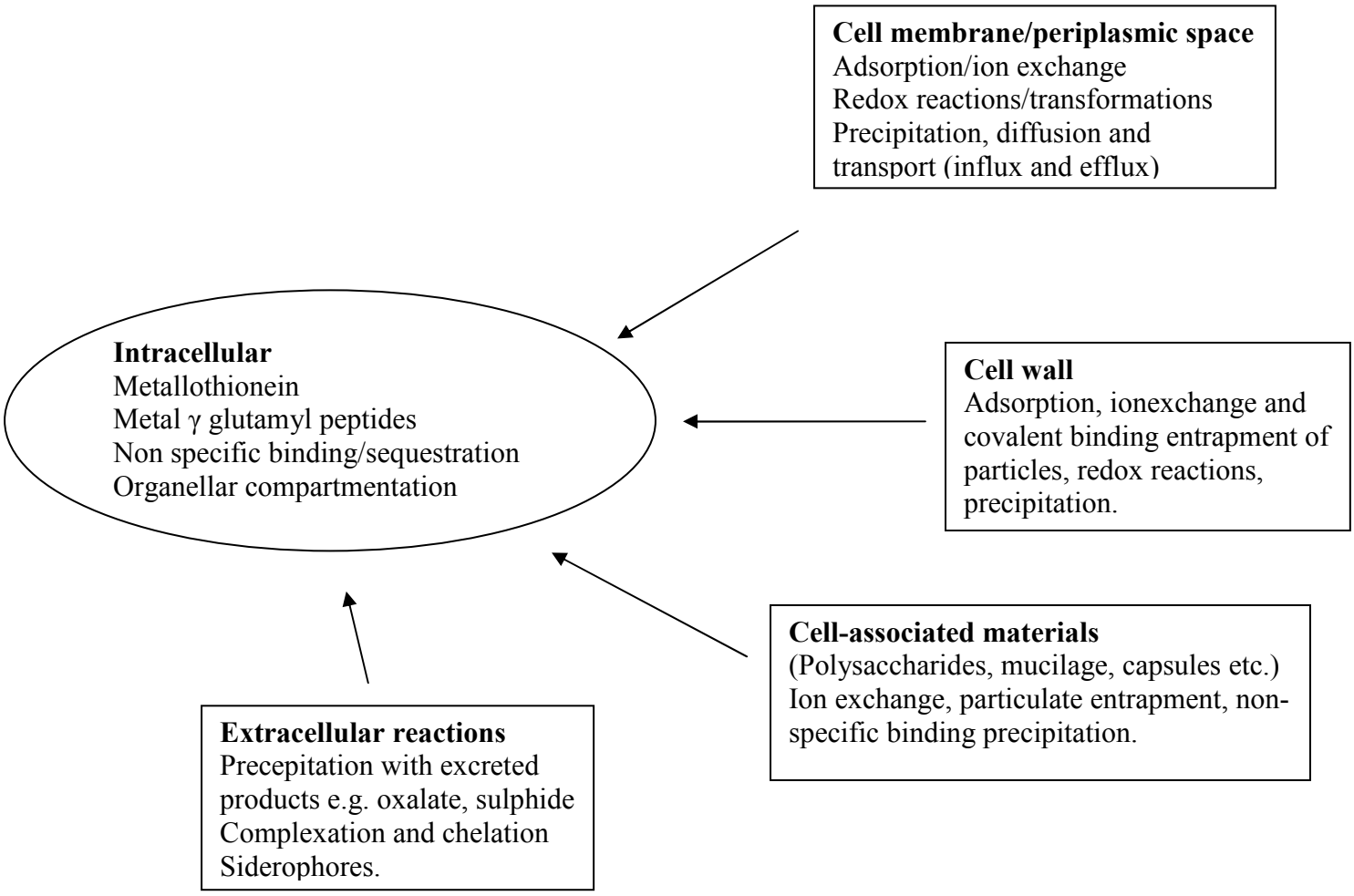

Figure 2.1: Bioremediation mechanisms by microorganisms (Thakur, 2006).

Transport of the metal across the cell membrane yields intracellular accumulation, which is dependent on the cell's metabolism. This means that this kind of accumulation may take place only with viable cells. It is often associated with an active defense system of the microorganism, which reacts in the presence of toxic metal. During non-metabolism dependent process metal uptake is by physico-chemical interaction between the metal and the functional groups present on the microbial cell surface. This is based on physical adsorption, ion exchange and chemical sorption, which is not dependent on the cells' metabolism. Cell walls of microbial biomass, mainly composed of polysaccharides, proteins and lipids have abundant metal binding groups such as carboxyl, sulphate,

phosphate and amino groups. This process i.e., non-metabolism dependent is relatively rapid and can be reversible (Kuyucak and Volesky, 1988). In the case of precipitation, the metal uptake may take place both in the solution and on the cell surface (Ercole, et al. 1994). Further, it may be dependent on the cell's' metabolism if, in the presence of toxic metals, the microorganism produces compounds that favor the precipitation process. Precipitation may not be dependent on the cells' metabolism, if it occurs after a chemical interaction between the metal and cell surface.

2.06.1 Transport across cell membrane

Heavy metal transport across microbial cell membranes may be mediated by the same mechanism used to convey metabolically important ions such as potassium, magnesium and sodium. The metal transport systems may become confused by the presence of heavy metal ions of the same charge and ionic radius associated with essential ions. This kind of mechanism is not associated with metabolic activity. Basically bioaccumulation by living organisms comprises of two steps. First, a metabolism independent binding takes place where the metals are bound to the cell walls followed by metabolism dependent intracellular uptake, whereby metal ions are transported across the cell membrane. (Costa, et.al., 1990; Gadd et.al., 1988; Huang et.al., 1990; Nourbaksh et.al., 1994)

2.06.2 Physical adsorption

In this category, physical adsorption takes place with the help of van der Waals' forces. (Kuyucak and Volesky 1988) hypothesized that uranium, cadmium, zinc, copper and cobalt biosorption by dead biomasses of algae, fungi and yeasts takes place through electrostatic interactions between the metal ions in solutions and cell walls of microbial cells. Electrostatic interactions have been demonstrated to be responsible for copper biosorption by bacterium *Zoogloea ramigera* and alga *Chiarella vulgaris* (Aksu et al. 1992), for Chromium biosorption by fungi *Ganoderma lucidum* and *Aspergillus niger.*

2.06.3 Ion Exchange

Cell walls of microorganisms contain polysaccharides and bivalent metal ions exchange with the counter ions of the polysaccharides. For example, the alginates of marine algae occur as salts of K+, Na+, Ca^2+, and Mg^2+. These ions can exchange with counter ions such as CO^2+, Cu^2+, Cd^2+ and Zn^2+ resulting in the uptake of heavy metals

(Kuyucak and Volesky 1988). The copper uptake by fungi *Ganoderma lucidium* (Muraleedharan and Venkobachr, 1990) and *Aspergillus niger* was also up taken by ion exchange mechanism.

2.06.4 Complexation

The metal removal from solution may also take place by complex formation on the cell surface after the interaction between the metal and the active groups. (Aksu et al. 1992) hypothesized that uptake of copper by C. *vulgaris* and Z. *ramigera* takes place through both adsorption and formation of coordination bonds between metals and amino and carboxyl groups of cell wall polysaccharides. Complexation was found to be the only mechanism responsible for calcium, magnesium, cadmium, zinc, copper and Mercury accumulation by *Pseudomonas syringae.* Microorganisms may also produce organic acids (e.g., citric, oxalic, gluonic, fumaric, lactic and malic acids), which may chelate toxic metals result in the formation of metallo-organic molecules. These organic acids help in the solubilisation of metal compounds and their leaching from their surfaces. Metals may be biosorbed or complexed by carboxyl groups found in microbial polysaccharides and other polymers.

2.06.5 Precipitation

Precipitation may be either dependent on the cellular metabolism or independent of it. In the former case, the metal removal from solution is often associated with active defense system of the microorganisms. They react in the presence of toxic metal producing compounds, which favor the precipitation process. In the case of precipitation not dependent on the cellular metabolism, it may be a consequence of the chemical interaction between the metal and the cell surface. The various biosorption mechanisms mentioned above can take place simultaneously.

2.07 Use of Recombinant bacteria for metal removal

Metal removal by adsorbents from water and wastewater is strongly influenced by physico-chemical parameters such as ionic strength, pH and the concentration of competing organic and inorganic compounds. Recombinant bacteria are being investigated for removing specific metals from contaminated water. For example a genetically engineered *E.coli,* which expresses Hg^{2+} transport system and metallothionin

(a metal binding protein), was able to selectively accumulate 8 mM Hg^{2+}/*g* cell dry weight. The presence of chelating agents Na^{+}, Mg^{2+} and Ca^{2+} did not affect bioaccumulation.

2.08 Biosorption and Bioaccumulation

Bioaccumulation is defined as the phenomenon of living cells; whereas, biosorption mechanisms are based on the use of dead biomass. Biosorption possesses certain inherent advantages over bioaccumulation processes, which are shown in the below.

Table-2.2: Differences between Biosroption and Bioaccumulation.

Features	Biosorption	Bioaccumulation
Cost	Usually low. Most biosorbents used were industrial, agricultural and other type of waste biomass. Cost involves mainly transportation and other simple processing charges.	Usually high. The process involves living cells and; hence, cell maintenance is cost prone.
pH	The solution pH strongly influences the uptake capacity of biomass. However, the process can be operated under a wide range of pH conditions.	In addition to uptake, the living cells themselves are strongly affected under extreme pH conditions.
Temperature	Since the biomass is inactive, temperature does not influence the process. In fact, several investigators reported uptake enhancement with temperature rise	Temperature severely affects the process.
Maintenance /storage	Easy to store and use	External metabolic energy is needed for maintenance of the culture
Selectivity	Poor. However, selectivity can be improved by modification/processing of Biomass	Better than biosorption
Versatility	Reasonably good. The binding sites can accommodate a variety of ions	Not very flexible. Prone to be affected by high metal/salt conditins.
Degree of uptake	Very high. Some biomasses are reported to accommodate an amount of toxicant nearly as high as their dry weight	Because living cells are sensitive to high toxicant concentration, uptake is usually low.

Rate of uptake	Usually rapid. Most biosorption mechanisms are rapid.	Usually slower than biosorption. Since intracellular accumulation is time consuming.
Toxicant affinity	High under favorable conditions.	Depends on the toxicity of the pollutant.
Regeneration and reuse	High possibility of biosorbent regeneration, with possible reuse over a number of cycles.	Since most toxicants are intracellularly accumulated, the chances are very limited.
Toxicant recovery	With proper selection of elutant, toxicant recovery is possible. In many instances, acidic or alkaline solutions proved an efficient medium to recover toxicants.	Even if possible, the biomass cannot be utilized for next cycle.

2.08.1 Biosorbent materials

Any biological material which exhibits its affinity and concentrates the heavy metals even in very dilute aqueous solutions is called as biosorbent material. This biological material may be dead or attenuated and the dead cells are called as 'magical granules'. Strong biosorbent behavior of certain micro-organisms towards metallic ions is a function of the chemical make-up of the microbial cells. Some types of biosorbents would be broad range, binding and collecting the majority of heavy metals with no specific activity, while others are specific for certain metals. Some laboratories have used easily available biomass whereas others have isolated specific strains of microorganisms and some have also processed the existing raw biomass to a certain degree to improve their biosorption properties. Recent biosorption experiments have focused attention on waste materials, which are by-products or the waste materials from large-scale industrial operations. For e.g. the waste mycelia available from fermentation processes, olive mill solid residues (Pagnanelli, et al 2002), activated sludge from sewage treatment plants (Hammaini et al. 2003), biosolids (Norton et al 2003), aquatic macrophytes (Keskinkan et aI. 2003), etc. Norton et aI. 2003 used dewatered waste activated sludge from a sewage treatment plant for the biosorption of zinc from aqueous solutions. The adsorption capacity was determined to be 0.564 mM/g of biosolids. The use of biosolids for zinc adsorption was favorable compared to the bioadsorption rate of 0.299 mM/g by the seaweed *Durvillea potatorum* (Aderhold et aI. 1996). Keskinkan et al. 2003 studied the

adsorption characteristics of copper, zinc and lead on submerged aquatic plant *Myriophyllum spicatum.* Pagnanelli, et al 2002 have carried out a preliminary study on the 'Use of olive mill residues as heavy metal sorbent material. The results revealed that copper was maximally adsorbed in the range of 5.0 to 13.5 mg/g under different operating conditions. The simultaneous biosorption capacity of copper, cadmium and zinc on dried activated sludge (Hammaini et al. 2003) were 0.32mmoI/g for metal system such as Cu-Cd; 0.29mmoI/g for Cu-Zn and 0.32mmoI/g for Cd-Zn. The results showed that the biomass had a net preference for copper followed by cadmium and zinc.

2.08.2 Bacterial biosorption

Early in 1980 it was witnessed that the capability of some microorganisms to accumulate metallic elements. Numerous research reports have been published from toxicological points of view, but these were concerned with the accumulation due to the active metabolism of living cells, the effects of metal on the metabolic activities of the microbial cell and the consequences of accumulation on the food chain (Volesky, 1987). However, further research has revealed that inactive/dead microbial biomass can passively bind metal ions via various physicochemical mechanisms. With this new finding, research on biosorption became active, with numerous biosorbents of different origins being proposed for the removal of metals. Researchers have understood and explained that biosorption depends not only on the type or chemical composition of the biomass, but also on the external physicochemical factors and solution chemistry. Many investigators have been able to explain the mechanisms responsible for biosorption, which may be one or combination of ion exchange, complexation, coordination, adsorption, electrostatic interaction, chelation and micro precipitation (Vegliò and Beolchini, 1997; Volesky and Schiewer, 1999). Table-2.3 summarizes some of the important results of metal biosorption using bacterial biomasses. A direct comparison of experimental data is not possible, due to different systematic experimental conditions employed (pH, temperature, equilibrium time and biomass dosage). However, Table-2.3 provides basic information to evaluate the possibility of using bacterial biomass for the uptake of metal ions. Also, it should be noted that Table-2.3 is only comprised of biosorption studies that employed either inactive or dead bacterial biomasses.

Table-2.3: Different microorganisms for various metal biosorption.

Metal	Organism
Aluminum	*Chryseomonas luteola*
Chromium (VI)	*Aeromonas caviae* *Bacillus coagulans* *Bacillus licheniformis* *Bacillus megaterium* *Bacillus thuringiensis* *Chryseomonas luteola* *Pseudomonas sp* *Staphylococcus xylosus* *Zoogloea ramigera*
Copper	*Bacillus sp. (ATS-1)* *Bacillus subtilis IAM 1026* *Enterobacter sp.J1* *Micrococcus luteus IAM 1056* *Pseudomonas aeruginosa PU21* *Pseudomonas cepacia* *Pseudomonas putida* *Pseudomonas putida sp* *Pseudomonas putidaCZ1* *Pseudomonas stutzeri IAM 12097* *Sphaerotilus natans* *Sphaerotilus natans* *Streptomyces coelicolor* *ThioBacillus ferrooxidans* *ThioBacillus ferrooxidans*
Cadmium	*Aeromonas caviae* *Bacillus circulans* *Enterobacter sp. J1* *Pseudomonas aeruginosa PU21* *Pseudomonas putida* *Pseudomonas sp.* *Staphylococcus xylosus* *Streptomyces pimprina* *Streptomyces rimosus*
Iron (III)	*Streptomyces rimosus*
Lead	*Bacillus sp. (ATS-1)* *Corynebacterium glutamicum* *Enterobacter sp. J1* *Pseudomonas aeruginosa PU21* *Pseudomonas aeruginosa PU21* *Pseudomonas putida* *Pseudomonas putida* *Streptomyces rimosus* *Streptoverticillium cinnamoneum*

Mercury	*Bacillus sp.*
Nickel	*Bacillus thuringiensis* *Streptomyces rimosus*
Palladium	*Desulfovibrio desulfuricans* *Desulfovibrio fructosivorans* *Desulfovibrio vulgaris*
Platinum	*Desulfovibrio desulfuricans* *Desulfovibrio fructosivorans* *Desulfovibrio vulgaris*
Thorium	*Arthrobacter nicotianae IAM 12342* *Bacillus licheniformis IAM 111054* *Bacillus megaterium IAM 1166* *Bacillus subtilis IAM 1026* *Corynebacterium equi IAM 1038* *Corynebacterium glutamicum IAM 12435* *Micrococcus luteus IAM 1056* *Nocardia erythropolis IAM 1399* *Zoogloea ramigera IAM 12136*
Uranium	*Uranium Arthrobacter nicotianae IAM* *Bacillus licheniformis IAM 111054* *Bacillus megaterium IAM 1166* *Bacillus subtilis IAM 1026* *Corynebacterium equi IAM 1038* *Corynebacterium glutamicum IAM 12435* *Micrococcus luteus IAM 1056* *Nocardia erythropolis IAM 1399* *Zoogloea ramigera IAM 12136*
Zinc	*Aphanothece halophytica* *Pseudomonas putida* *Pseudomonas putida CZ1* *Streptomyces rimosus* *Streptomyces rimosus* *Streptoverticillium cinnamoneum* *ThioBacillus ferrooxidans* *ThioBacillus ferrooxidans*

2.08.3 Mechanism of bacterial biosorption

The bacterial cell wall is the first component that comes into contact with metal ions where the solutes can be deposited on the surface or within the cell wall structure (Beveridge and Murray, 1976; Doyle et al., 1980). Since the mode of solute uptake by dead/inactive cells is extracellular, the chemical functional groups of the cell wall play vital roles in biosorption. Due to the nature of the cellular components, several functional groups are present on the bacterial cell wall, including carboxyl, phosphonate, amine and

hydroxyl groups (Doyle et al., 1980; van derWaal et al., 1997). As they are negatively charged and abundantly available, carboxyl groups actively participate in the binding of metal cations. Several dye molecules, which exist as dye cations in solutions, are also attracted towards carboxyl and other negatively charged groups. Golab and Breitenbach (1995) indicated that the carboxyl groups of the cell wall peptidoglycan of Streptomyces pilosus were responsible for the binding of copper. Also, amine groups are very effective at removing metal ions, as it not only chelates cationic metal ions, but also adsorbs anionic metal species or dyes via electrostatic interaction or hydrogen bonding. Kang et al. (2007) observed that amine groups protonated at pH-3 and attracted negatively charged chromate ions via electrostatic interaction. Vijayaraghavan and Yun (2007b) confirmed that the amine groups of *C. glutamicum* were responsible for the binding of reactive dye anions via electrostatic attraction. In general, increasing the pH increases the overall negative charge on the surface of cells until all the relevant functional groups are deprotonated, which favors the electrochemical attraction and adsorption of cations. Anions would be expected to interact more strongly with cells with increasing concentration of positive charges, due to the protonation of functional groups at lower pH values. The solution chemistry affects not only the bacterial surface chemistry, but the metal/dye speciation as well. Metal ions in solution undergo hydrolysis as the pH increases. The extent of which differs at different pH values and with each metal, but the usual sequence of hydrolysis is the formation of hydroxylated monomeric species, followed by the formation of polymeric species, and then the formation of crystalline oxide precipitates after aging (Baes and Mesmer, 1976). For example, in the case of nickel solution, López et al. (2000) indicated that within the pH range from 1 to 7, nickel existed in solution as Ni^{2+} ions (90%); whereas at pH 9, Ni^{2+} (68%), $Ni_4OH_4{}^{4+}$ (10%) and $Ni(OH)^{+}$ (8.6%) co-existed. The different chemical species of a metal occurring with pH changes will have variable charges and adsorbability at solid–liquid interfaces. In many instances, biosorption experiments conducted at high alkaline pH values have been reported to complicate evaluation of the biosorbent potential as a result of metal precipitation (Selatnia et al., 2004b; Iqbal and Saeed, 2007).

2.09 Choice of metal for biosorption process

The appropriate selection of metals for biosorption studies is dependent on the angle of interest and the impact of different metals, on the basis of which they would be divided into four major categories: (I) Toxic heavy metals (II) Strategic metals (III) Precious metals and (IV) Radio nuclides. In terms of environmental threats, it is mainly categories (I) and (IV) that are of interest for removal from the environment and/or from point source effluent discharges. Apart from toxicological criteria, the interest in specific metals may also be based on how representative their behaviour may be in terms of eventual generalization of results of studying their biosorbent uptake. The toxicity and interesting solution chemistry of elements such as Chromium, Arsenic and selenium make them interesting to study. Strategic and precious metals though not environmentally threatening are important from their recovery point of view.

2.10 Objectives of present study

1. To study the capabilities of gram negative *Pseudomonas aeruginosa* and gram positive *Bacillus subtilis* cell surfaces in heavy metal biosorption process.
    - Simultaneously studying the factors that affect the biosorption process.
    - To study the factors that affects the biosorption of combination of metal pollutants.
2. To study the capabilities of combined *Pseudomonas aeruginosa* and *Bacillus subtilis* cell surfaces in heavy metal biosorption, where solution contains combinations of metal pollutants.

Chapter 3

Materials and methods

3.1 Materials

3.1.1 Chemicals

The metal salts used in this work are $K_2Cr_2O_7$(MG7M571737) and $HgCl_2$ obtained from Merck specialIties Pvt Limited, Mumbai, India, $Na_2HAsO_4.7H_2O$ (Art.5770) obtained from Loba Chemie Pvt ltd, India as respective source of metal ions Chromium, mrecury and Arsenic. Nutrient medium (M002-500G) for subculturing, Nutrient Agar (M001-500G) for slant culture, Hydrochloric acid (HCl) and sodium hydroxide (NaOH) for adjusting pH obtained from Himedia, India.

The Bromfield medium for culturing composed per liter (pH-7) each of KH_2PO_4 - 0.50g (Art.5429) from Loba Chemie pvt Ltd, India, $MgS0_4.7H_20$- 0.20g (ML7M573186) from Merck specialIties Pvt Ltd, $(NH_4)_2SO_4$ -1.0g (MK7M572693), Yeast extract -0.15g (MK6M562910) and Sucrose 20.0-g (MB8M580322) obtained from Himedia, India. Actone (SJ7SF71144) and Diphenyl carbazide (MC7M570666) obtained from Merck specialIties Pvt Ltd, Mumbai, India, used for Chromium analysis by UV–Vis. spectrophotometer (Jasco, Japan, V–530) at an absorbance of 540nm. Ethanol (15005-51) from Hong Young Chemicals Pvt Ltd, Mumbai, India. All the chemicals were used as received. Ultra pure water (Sartorius, Germany) was used for the experiments of 18.2 mΩ resistivity and pH 6.8 – 7.

3.1.2 Microorganisms

Gram negative and gram positive microorganisms *Pseudomonas aeruginosa 2053, Bacillus subtilis 2010* for the study were purchased from National Collection of Industrial Microorganisms (NCIM), Pune. Each strain maintained in the nutrient medium and appropriate proportions used for the experiment. Standard sterile techniques were used for inoculation of cultures. Medium used for the microorganism and all the glassware were properly sterilized autoclaved at 15 lb/in^2 pressure and 121^0C for 30 minutes.

3.2 Methods

3.2.1 Preparation of Metal solutions

Individual Metal solutions preparation

Different metal concentrations were prepared by dissolving $K_2Cr_2O_7$, $Na_2HAsO_4.7H_2O$ and $HgCl_2$ in double distilled water to get metal concentrations of 5, 10, 15, 20, 30 mg/L. A stock solution of 1000 mg/L was prepared all other concentrations are obtained from it.

Binary metal solutions preparation

Chromium-Mercury and Mercury-Arsenic binary metal solutions were prepared using 10 mg/L each and mixed in equal proportions. All the metal solutions prepared in sterilized galssware obtained from Borosil, India. Prior to experiment all the glass ware treated with 0.1 M HCl before and after the biosorption experiments to avoid binding of metals to it.

3.2.2 Biosorbent preparation

1000 ml of Nutrient medium was prepared with standard composition in a conical flask. The pH for the medium was adjusted accordingly and then the media was sterilized at 15 lb/in^2 pressure and 121^{0}C for 30 minutes. Nutrient agar medium Himedia was prepared, autoclaved and allowed to cool. Loop full of bacterial culture was taken and streaked on the agar plate to obtain more colonies. They are later transferred to nutrient broth and grown on specific media (Bromifield medium-*Bacillus subtilis*; Cetrimide medium-*Pseudomonas aeruginosa*) for subculture. 100 ml of sterilized culture media was transferred to 250 ml Erlenmeyer flask. The media was allowed to cool and then the 100µl microbial solution was inoculated into the medium in laminar air flow chamber.

The inoculated flasks were incubated in an orbital shaker (Metrex scientific instruments, India) at 250 rpm at 32^{0}C for 2 days to obtain the biomass. Mixed cultures were prepared by adding equal amounts of individual cultures. Biomass was harvested from the medium by centrifugation at 9000 rpm for 10 min. The supernatant was discarded and the cells were re-suspended in purified water for washing and again centrifuged as above to make sure that no media remain on the cell surface. The biomass was heat killed in a conventional hot air oven at 60^{o}C for 24 hrs. This biomass was used

for the sorption experiments. Both the biomasses were added in equal amounts for sorption experiments with mixed culture.

3.2.3 Biosorption experiment

Different concentrations of biomass (pure/mixed cultures) were combined with 100 ml of metal solution in 250 ml Erlenmeyer flask. The flasks were placed on a shaker with a constant speed of 300 rpm and left to equilibrate. Samples were collected at pre-defined time intervals, centrifuged as above and the amount of metal in the supernatant was determined.

3.2.4 Instruments used

- UV-Visible spectrophotometer, Jasco, V–530, Japan.
- Atomic absorption spectrophotometer, Perkin Elmer AAnalyst 200, Singapore.
- Centrifuge, Hettich, Zentrifugen, Universal 320 R, Germany.
- Autoclave, Testmaster, Kolkata, India.
- Shaker incubator, Metrex scientific instruments, New Delhi, India.
- Hot air oven, Bhattacharya & Co DTC 72S1, Kolkata, India.

3.2.5 Anlystical estimation of Chromium (VI)

A 0.25% w/v solution of diphenyl carbazide was prepared in 50% acetone. 15 ml each of the sample solutions, containing various concentrations of Cr (VI) were pipetted out into 25ml standard flasks. To this 2ml of 3M H_2SO_4 was added followed by l ml of diphenyl carbazide and the total volume was made upto 25 ml using deionised, double distilled water such that the final concentrations were in the range of 0.15 to 0.3 ppm. Chromium concentration estimated by the intensity of the colour complex formed was measured using a UV-visible spectrophotometer. The absorbance was measured against a reagent blank at 540-nm wavelength maximum. A linear plot was obtained indicating adherence to the Beer Lambert's law in the concentration range studied.

$$\% \text{ Biosorption} = \frac{(\text{Initial} - \text{Final metal concentration})}{(\text{Initial metal concentration})} * 100 \qquad (3.1)$$

3.2.6 Analytical estimation of Mercury and Arsenic

The concentrations of Arsenic in the samples were measured by AAS (atomic absorption spectrophotometer, Perkin Elmer AAnalyst 200) in flame at a wave length 197.2 nm. The concentration of Mercury was measured by AAS at 253.7 nm with sodium tetrahydroborate as reductant. Each adsorption kinetics experiment was carried out twice, and the average was used in this work.

3.2.7 Biosorption studies

Biomass was harvested from the medium by centrifugation at 9000 rpm for 10 min. The supernatant was discarded and the cells were re-suspended in purified water for washing and again centrifuged as above to make sure that no media remain on the cell surface. The biomass was heat killed in a conventional hot air oven at 60^{o}C for 24 hrs. This biomass was used for the sorption experiments.

Biosorption studies were done using biomass as a function of various parameters such as

a) pH

b) Biomass concentration

c) Temperature

d) Time

e) Initial metal concentration

3.2.7.1 Effect of pH

The metal sorption monitored for pH range 1 to 7. NaOH and HCL were used as pH regulators. 1 mg/ml biomass was dispersed in 100 ml of the solution containing 10mg/L of each metal concentration. All flasks were maintained at different pH values ranging from 1 to 7 for about 12 hours. Solutions were centrifuged as above and the supematant was analysed for the residual concentrations of the metal ions. The final pH values have been plotted.

3.2.7.2 Effect of biomass concentration

Biomass was centrifuged at 9000 rpm and different weights of the biomass ranging from 0.5 to 3 mg/ml were dispersed in solutions containing the 10 mg/L metal concentration. The solutions were adjusted to the optimum pH in which maximum biosorption of the metal ion occurred. Flasks were left for equilibration. The solutions

were later centrifuged at 9000 rpm and the metal ion concentrations were determined using the procedures described earlier.

3.2.7.3 Effect of temperature

Optimum biomass concentration with optimum pH was used to monitor the temperature effect on biosorption. Experiments were carried out at different temperatures from10-50^{o}C for each culture and kept on rotary shaker at 240 rpm. The samples were allowed to attain equilibrium. The sample collected at regular intervals as above and analyzed for metal concentration.

3.2.7.4 Effect of time

The cell pellet dispersed in metal solution of 10 mg/L concentration with a working volume of 100 ml. the experiment was carried out at the optimum pH system. Flasks were allowed to attain equilibrium on rotary shaker at 240 rpm and samples were collected at regular time intervals. Centrifugation at 9000 rpm was done and the supernatant was analysed for the residual metal content.

3.2.7.5 Effect of initial metal concentration

Biosorption experiments were conducted by taking different initial metal concentrations by fixing all the parameters such as biomass concentration, pH, temperature and time. Metal solutions were prepared as stated in section 3.21. With increase in metal concentration (5 to 30 mg/L) percentage biosorption was observed.

3.2.8 Adsorption isotherms

The optimum biomass of each culture was dispersed in a desired concentration ranging from 5 mg/L to 30 mg/L for each metal. In all these cases the initial pH was adjusted to that of the optimum value, namely 3, 5, 6 for Chromium, Mercury and Arsenic respectively. The flasks were incubated for their respective period of time (50, 25, 15 for Mercury, Chromium, Arsenic), at the end of which the residual concentrations were determined.

Data evaluation

The amount of metal bound by the biosorbents was calculated as follows

$$Q = v (C_i - C_f)/m \qquad (3.2)$$

Where Q is the metal uptake (mg metal per g biosorbent), v is the liquid sample volume (ml), Ci is the initial concentration of the metal in the solution (mg/l), Cf is the

final (equilibrium) concentration of the metal in the supernatant (mg/l) and m is the amount of the added biosorbent on the dry basis (mg).

The Langmuir model,

$$Q = Q_{max}\, bC_f / 1+bC_f \quad (3.3)$$

Where Q_{max} is the maximum metal uptake under the given conditions, b a constant related to the affinity between the biosorbent and sorbate.

Linearized Langmuir model

$$1/Q = 1/Q_{max}\,(1/b\,C_f + 1) \quad (3.4)$$

The Freundlich Model,

$$Q = k\,C_f^{(1/n)} \quad (3.5)$$

Where k and n are Freundlich constant, which correlated to the maximum adsorption capacity and adsorption intensity, respectively.

Linearized Freundlich equation

$$\text{Log } Q = \text{Log } k + 1/n \log C_f. \quad (3.6)$$

3.2.9 Rate kinetics

As aforementioned, a lumped analysis of adsorption rate is sufficient to practical operation from a system design point of view. The commonly employed lumped kinetic models, namely a) the pseudo-first-order equation b) the pseudo-second-order equation are presented below (Yang and Duri, 2005).

The pseudo first-order and pseudo-second-order kinetic models assume that adsopriton is a pseudo-chemical reaction process and the adsorption rate can be determined respectively by the first-order and second-order reaction rate equations,

$$\frac{dq_t}{d_t} = k_{1(}q_e - q_t) \quad (3.7)$$

$$\frac{dq_t}{d_t} = k_{2(}q_e - q_t)^2 \quad (3.8)$$

Where q_e (mg g^{-1}) is the solid phase concentration at equilibrium, q_t (mg g^{-1}) is the average solid phase concentration at time t (min), and k_1 (min^{-1}) and k_2 (g mg^{-1} min^{-1}) are the pseudo-first-order and pseudo-second order rate onstants, respectively. The above

equations represent initial value problems and have analytical solutions when combined with the initial condition $t = 0$, $q_t = 0$.

The solutions for equations (3.7) and (3.8) are as follows:

$$\ln(qe - qt) = \ln(qe) - k_1 t \tag{3.9}$$

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e} \tag{3.10}$$

If the adsorption follows the pseudo-first order rate equation, a plot of ln ($q_e - q_t$) against time t should be a straight line. Similarly, t/q_t should change linearly with time t if the adsorption process obeys the pseudo-second order rate equation. Available studies have shown that the pseudo-second order rate equation is reasonably good fit of data over the entire fractional approach to equilibrium and theirfore has been employed extensively in the study of adsorption kinetics (Wu et al., 2001; Chang et al., 2003).

However, it is not uncommon to observe multi linearity on the ln (q_e-q_t) – t plot or t/q_t – t plot. The trend is usually such that the rate constant decreases with time or more specially decreases with increase in solid phase concentration.

Chapter 4

Biosorption of Mercury using individual and mixed cultures of *Bacillus subtilis* and *Pseudomonas aeruginosa*

4.1 Results and discussion

4.1.1 Biosorption studies using attenuated cells of Pseudomonas aeruginosa

In the investigation carried out so far, the attenuated cells of *Pseudomonas aeruginosa* were used for the biosorption of Mercury. The parameters influencing the biosorption of Mercury using this single culture of *Pseudomonas aeruginosa* are studied. Futher more the effects of these parameters are discussed below:

4.1.1.1 Effect of pH

The most important single parameter influencing the sorption capacity is the pH of the adsorption medium. (Goyal et al., 2003). The influence of pH on the percentage sorption of Mercury is depicted in the Figure 4.1. The sorption increased from 50% at pH 3 to 98% at 5 and significantly decreased with increase of pH. But at pH 6 and 7 it was around 40% and 20%. Same condition was observed at lower pH, like at pH 2 it was around 30%. The pH trend observed in this case is shown in Figure 4.1; from this study we can conclude that at pH 5 for *Pseudomonas aeruginosa* maximum percent of biosorption occured. The fluctuation beyond this optimum pH 5 was due to decrease of low availability of surface for sorption at low pH and formation of metal hydroxide and other metal-ligand complexes significantly reduce the amount of metal ions sorbed at high pH (Vijayaraghavan and Yun, 2008).

4.1.1.2 Effect of biomass concentration

The influence of biomass concentration on the percentage sorption of Mercury is depicted in Figure 4.2. To achieve the maximum biosorption capacity of the biosorbent for Mercury, the biomass concentration was varied from 0.5 to 3 mg/ml and it was found that a concentration of 0.5 mg/ml was adequate for maximum percentage of Mercury

biosorption under the reported experimental conditions. These findings are shown in Figure 4.2. It is also seen from this Figure that a further increase in biomass does not affect the sorption percentage greatly. This may be due to the unavailability of binding sites to the metal and also due to the blockage of binding sites with excess biomass. In this study it was observed that at 0.5 mg/ml concentration showed highest sorption percentages (Vijayaraghavan and Yun, 2008).

4.1.1.3 Effect of temperature

In the studies of biosorption using attenuated cells of *Pseudomonas aeruginosa* it was observed that the temperature range between 24^{o}C to 32^{o}C was found to be favorable than that of the lower or higher temperatures. The influence of temperature is depicted in Figure 4.3. Maximum sorption of around 98% was seen at 32^{o}C. In these experiments there was an increase in sorption percentage with increase in the temperature till 32^{o}C. A gradual decrease in sorption percentage was observed after that. This is because of the shrinkage of cells at higher and lower temperatures which reduces the surface area of contact (Vijayaraghavan and Yun, 2008). From this we can conclude that the temperature 34^{o}C was favorable for biosorption of Mercury using *Pseudomonas aeruginosa*.

4.1.1.4 Effect of contact time

Here the optimum biomass concentration from Figure 4.2 taken for *Pseudomonas aeruginosa* and its time to reach maximum sorption was monitored. The adsorption experiments of Mercury were carried out for different contact times with a fixed adsorbent dose of 0.5 mg/ml concentration at pH 5 at 32^{o}C. The results are plotted in Figure 4.4, which indicate that maximum sorption attained at 60 min for Mercury.

4.1.2 Biosorption studies of Mercury using attenuated cells of Bacillus subtilis

In the investigation carried out so far, the attenuated cells of *Bacillus subtilis* were used for the biosorption of Mercury. The parameters affecting the biosorption of Mercury using single culture of *Bacillus subtilis* were studied. The effects of these parameters are discussed below.

4.1.2.1 Effect of pH

The biosorption of Mercury was studied over a pH range of 1 to 7 and the results are given in Figure 4.1. The maximum percent of sorption took place at pH 5. It is also apparent from this Figure that the sorption rises from pH 4 to 5 and then starts to decrease. The decrease of sorption percent at higher pH may be due to decrease in solubility of metal complexes sufficiently allowing precipitation, which may complicate the sorption process (Vijayaraghavan and Yun, 2008).

4.1.2.2 Effect of biomass concentration

To achieve the maximum biosorption capacity of the biosorbent for Mercury, the biomass concentration was varied from 0.5 to 3 mg/ml and it was found that a concentration of 2.5 mg/ml was sufficient for maximum biosorption of around 78%. These findings are shown in Figure 4.2 and a perusal of this Figure indicates that the sorption percentage increases from 0.5 to 2.5 mg/ml. It is also seen from this Figure that a further increase in biomass does not affect the sorption percentage greatly.

4.1.2.3 Effect of temperature

The biosorption studies were carried out at different temperatures from 10^{o}C to 50^{o}C and the results of these experiments are represented in the Figure 4.3. It is also observed that the maximum sorption around 78% occurred at 32^{o} C. These findings indicate that the sorption percentage increased with increase in temperature up to 32^{o}C. There was a decrease in sorption percentage with further increase in temperature. This may be due to the shrinkage of cells at higher temperature.

4.1.2.4 Effect of contact time

The studies at different contact times help in determining the sorption capacities of biomass at varying time intervals. The results are plotted in the Figure 4.4. These experiments were carried out by keeping biosorbent concentration fixed at optimum temperature and pH. It was observed that sorption percentage increased with increase in time up to 40 min. It showed a sorption of 78% at 40 min and remained almost constant with minute fluctuations.

4.1.3 Biosorption studies using mixed cultures of Pseudomonas aeruginosa and Bacillus subtilis

Influence of different parameters on the heavy metal biosorption by mixed culture of gram positive - gram negative surfaces and the following investigations were conducted.

4.1.3.1 Effect of pH

pH controls the metal ion dissolution and the magnitude of the electrostatic charge in the medium (Vijayaraghavan and Yun, 2008). The percent of metal sorption varies with pH of the medium. The experimental results of Mercury sorption using mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) at varying pH ranges was shown in the Figure 4.1. Effect of pH on biosorption was studied over a range of 1 to 7. Mixed cultures showed high sorption around 90% at pH 5. Figure 4.1 shows a decrease in sorption percentage with further increase in pH.

4.1.3.2 Effect of biomass concentration

Various amounts ranging from 0.5 to 3 mg/ml of mixed biomass were taken and biosorption of Mercury was observed at fixed pH (pH 5). The Figure 4.2 depicts the effect of biomass concentration on sorption percentage of Mercury. It was observed that biosorption percentage increased with increase in biomass. Significantly high biosorption around 90% was achieved at 2 mg/ml and this biomass concentration was chosen for all further studies.

4.1.3.3 Effect of temperature

Biosorption studies of Mercury using mixed cultures were carried out at different temperatures ranging from 10^{o}C to 50^{o}C. The effect of temperature on metal sorption was presented in Figure 4.3. The percentage of metal sorpted was increased from 10^{o}C to 32^{o}C and then showed decrease in sorption percentage with increase in temperature. Figure 4.3 shows a maximum percent of sorption around 90% achieved at 32^{o}C at fixed pH and biomass concentrations.

4.1.3.4 Effect of contact time

Biosorption experiments were carried out for different contact times at fixed pH, biomass concentration and temperature. The Figure 4.4 depicts the percent sorption with time. The sorption percentage of the metal increased with time. A sorption of 90% was

reached at 40 min. The sorption of metal was rapid in the initial stages of contact time and gradually decreased with lapse of time until saturation.

4.1.3.5 Rate kinetics

In order to determine a suitable kinetic model, the adsorption data was fitted into first order and second order kinetics (kinetic model described in Chapter 3). The first order equation was plotted for ln (q_e-q_t) against t. The values of ln (q_e-q_t) were calculated from the kinetic data of Figure 4.6(a). The k_1 value was calculated from the slope of this plot. The value of k_1 was shown in Table-4.2.

The second order equation was plotted for t/q_t against t (Figure 4.6(b)). The values of q_e and k_2 are calculated from the slope and intercept of this plot. The values of q_e and k_2 are shown in Table-4.2. The correlation coefficient R^2 =0.746 for pseudo-first order and R^2= 0.982 for pseudo-second order kinetic equation states that pseudo-second order well fitted with experimental values.

By maintaining all the parameters at optimum levels the initial metal concentrations were varied (5, 10, 15, 20, 25 mg/L). The percentage sorption was decreased constantly with increase in initial metal concentration. The decline in the percentage biosorption was depicted in Figure 4.5.

4.1.3.6 Adsorption isotherms

The applicability of Langmuir and Freundlich models for mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* was tested (Figure 4.7(a), 4.7(b)). The coefficient of determination (R^2) for both models was mostly greater than 0.95 and close to 1. This indicates that both models adequately describe the experimental data of the biosorption of Mercury.

In the biosorption of Mercury by mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis*, most of the metal ions were sequestered very fast from the solutions in the first phase of contact time 40 minutes and almost no increase in the level of bound metal have been occurred after this time interval as shown in Figure 4.4. The sorption performance of the mixed biosorbent was studied under fixed environmental conditions. Biosorption equilibrium isotherms were plotted for metal uptake q against the residual metal concentration in the solution. The q verses C_f sorption isotherm relationship was mathematically expressed by linearized Langmuir and Freundlich models. The higher the

values of k and n; lower the value of b, the higher the affinity of the biomass (Asku et al. 1991; Jalali et al., 2002). Table-4.3 describes summaries of linear regression data for Langmuir and Freundlich isotherms for Mercury biosorption using attenuate mixed biomass. Langmuir and Freundlich constant k was obtained from the linear equations of both models. As indicated in the Table-4.3, the coefficients of determination (R2) of both models are 0.99 close to 1. In the Table-4.3 the values of K_f, n, Q_{max} and b were given.

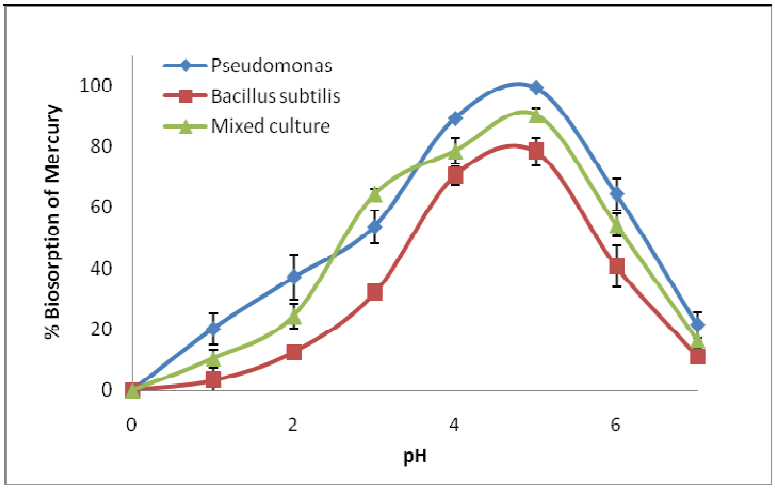

Figure 4.1: Effect of pH on percent mercury biosorption at 32 ^{o}C temperature, (0.5, 2.5, 2 mg/ml) biomass, (60, 40, 40 min) of contact time and 10 mg/L initial metal concentration for *Pseudomonas aeruginosa*, *Bacillus subtilis* and mixed culture (1:1).

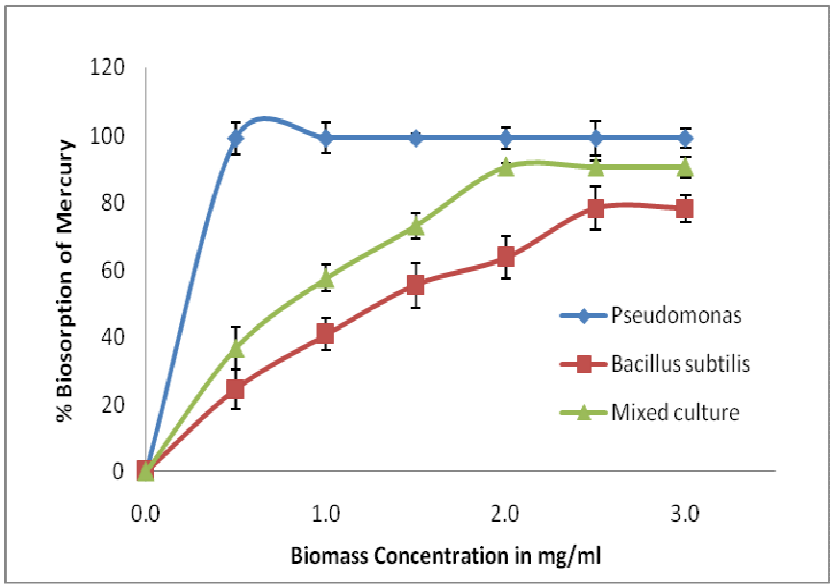

Figure 4.2: Effect of biomass concentration on percent mercury biosorption at 32 ^{o}C temperature, pH 5, contact time (60, 40, 40 min) and 10 mg/L initial metal concentration for *Pseudomonas aeruginosa*, *Bacillus subtilis* and mixed culture (1:1).

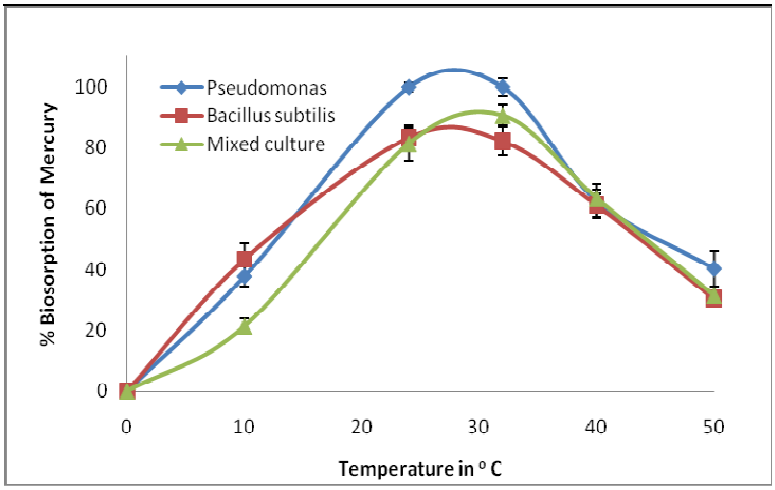

Figure 4.3: Effect of temperature on percent mercury biosorption at pH 5, (0.5, 2.5, 2 mg/ml) biomass, contact time (60, 40, 40 min) and 10 mg/L initial metal concentration for *Pseudomonas aeruginosa*, *Bacillus subtilis* and mixed culture (1:1).

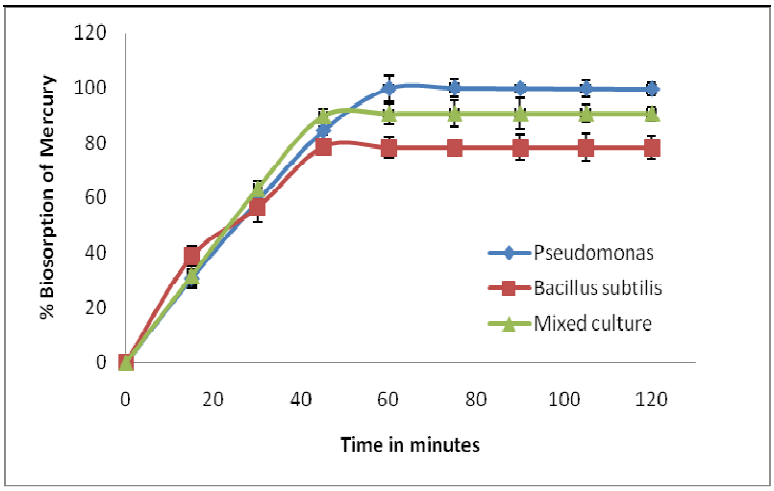

Figure 4.4: Effect of time on percent mercury biosorption at 32 ^{o}C temperature, pH 5, 0.5, 2.5, 2 mg/ml) biomass and 10 mg/L initial metal concentration for *Pseudomonas aeruginosa*, *Bacillus subtilis* and mixed biomass (1:1) concentration.

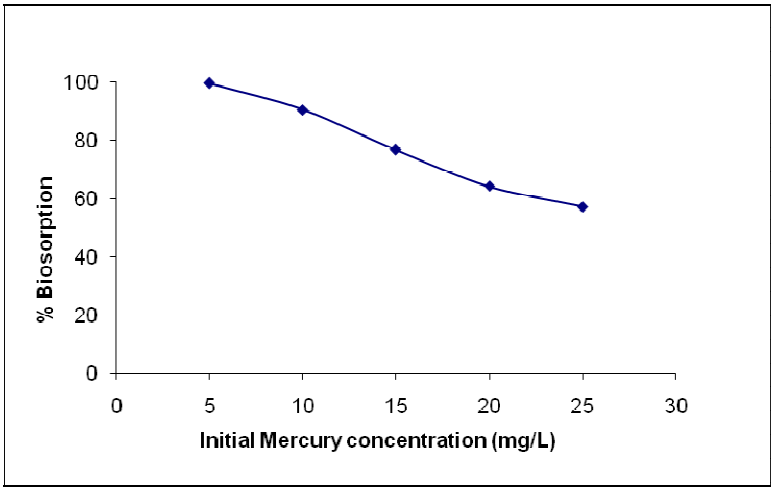

Figure 4.5: Effect of initial metal concentration at 2 mg/ml biomass, 32^{o}C temperature, pH 5, 40 min of contact time.

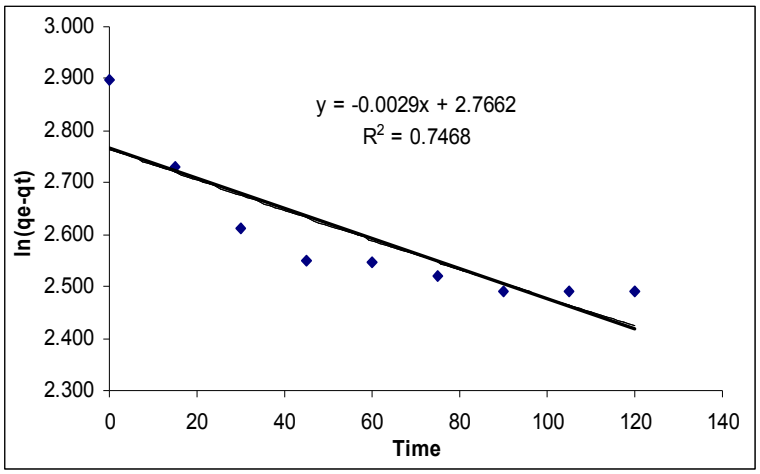

Figure 4.6(a): First order kinetics for Mercury by *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) mixed culture at 2 mg/ml biomass concnetration, 10 mg/L metal concentration, pH.5, 32^{o}c temperature.

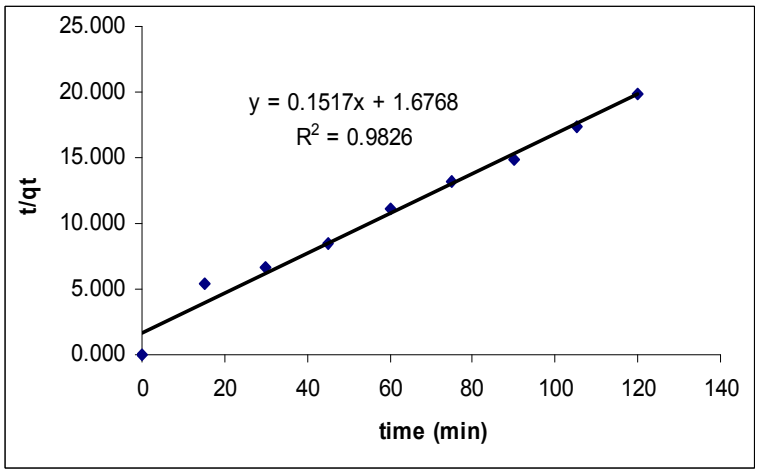

Figure 4.6(b): Second order kinetics for Mercury by *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) mixed culture at 2 mg/ml biomass concnetration, 10 mg/L metal concentration, pH.5, 32^{o}c temperature.

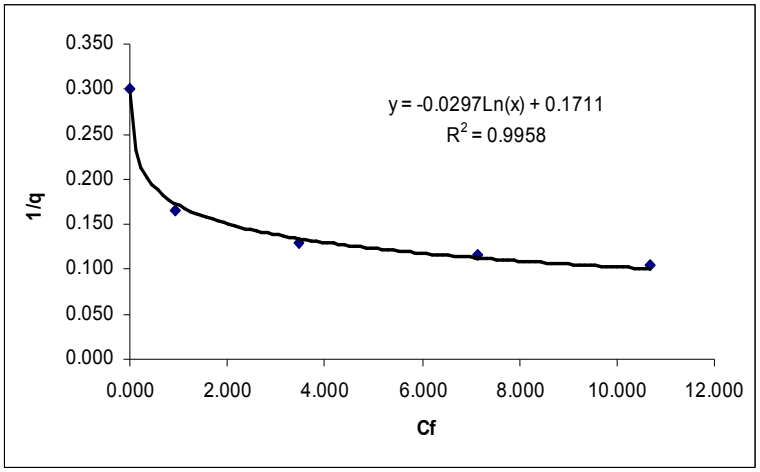

Figure 4.7(a): Adsopriton isotherm (Langmiur) for Mercury by *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) mixed culture at pH 5, 2 mg/ml biomass, 32^{o}C temperature, and 40 min of contact time.

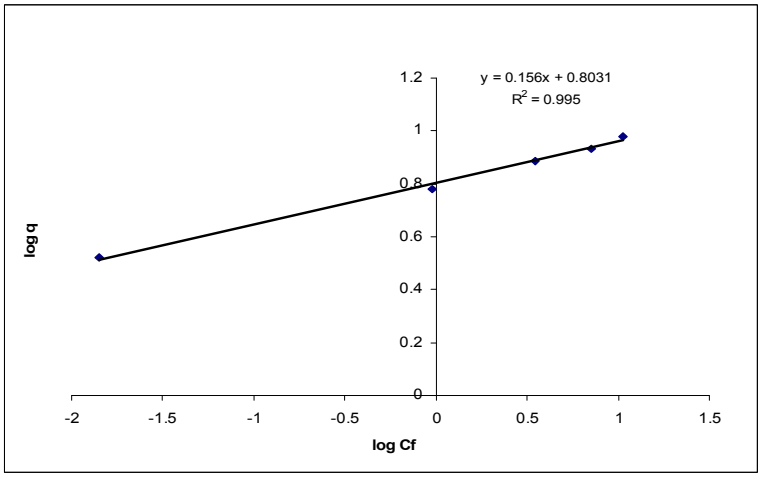

Figure 4.7(b): Adsopriton isotherm (Freundlich) for Mercury by *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) mixed culture at pH 5, 2 mg/ml biomass, 32^{o}C temperature, and 40 min of contact time.

Table-4.1: Kinetic data of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) mixed culture for Mercury.

Metal	Pseudo first order			Pseudo second order		
	K_1	q_e	R^2	K_2	q_e	R^2
Mercury	0.0029	15.89811	0.7468	0.031	6.591	0.9826

Table-4.2: Parameters of isotherm models for heavy metal Mercury.

Metal	Freundlich parameters			Langmuir parameters		
	K_f	1/n	R^2	q_m	b	R^2
Mercury	6.354	0.156	0.995	5.8445	-5.7045	0.9958

Chapter 5

Biosorption of Chromium using individual and mixed cultures of *Bacillus subtilis* and *Pseudomonas aeruginosa*

5.1 Results and Discussion

5.1.1 Biosorption studies using attenuated cells of Pseudomonas aeruginosa

In the investigation carried out so far, the attenuated cells of *Pseudomonas aeruginosa* were used for the biosorption of Chromium. The parameters affecting the Biosorption of Chromium using this single culture of *Pseudomonas aeruginosa* were studied. The effect of these parameters was discussed below.

5.1.1.1 Effect of pH

The pH value of the solution is an important factor that controls the sorption of Chromium. Figure 5.1 shows the pH of highest sorption efficiency (pH 3), the influence of pH on the percentage sorption of Chromium is depicted in the Figure 5.1. The percentage sorption increased from 55% at pH 2 to 62% at 3 and significantly decreased with increase in pH. Like at pH 6 it was around 20%. The pH trend observed in this case is shown in Figure 5.1 from this study we can conclude that at pH 3 *Pseudomonas aeruginosa* showed maximum percent of biosorption took place.

5.1.1.2 Effect of biomass concentration

Batch experiments were conducted to investigate the influence of biomass concentration on the percentage sorption of Chromium is depicted in Figure 5.2. To achieve the maximum biosorption capacity of the biosorbent for Chromium, the biomass concentration was varied from 0.5 to 3 mg/ml and it was found that a concentration of 1.5 mg/ml was sufficient for maximum percentage of Chromium biosorption. These findings are shown in Figure 5.2. It is also seen from this Figure that a further increase in biomass does not affect the sorption percentage greatly. This may be due to the unavailability of

binding sites to the metal and also due to the blockage of binding sites with excess biomass.

5.1.1.3 Effect of temperature

Effect of temperature on Chromium biosorption is presented in Figure 5.3. It was observed that the temperature 32^{o}C is favorable than that of the lower or higher temperatures. Good sorption percentage around 62% was observed at 32^{o}C. In these experiments there was an increase in sorption percentage with increase in the temperature but there was a gradual decrease with further increase in temperature. This is because of the shrinkage of cells in the higher and lower temperatures which reduces the surface area of contact.

5.1.1.4 Effect of contact time

The adsorption experiments of Chromium were carried out for different contact times with a fixed adsorbent dose of 1.5 mg/ml concentration at pH 3 and at 32^{o}C. The results were plotted in Figure 5.4. The sorption percentage of metal increased with increase in contact time. The equilibrium time was 30 min for Chromium (62%).

5.1.2 Biosorption studies using attenuated Bacillus subtilis

In the studies carried out so far, the attenuated cells of *Bacillus subtilis* were used for the biosorption of Chromium. The parameters affecting the Biosorption of Chromium using single culture of *Bacillus subtilis* were studied. Effects of these parameters are discussed below:

5.1.2.1 Effect of pH

The pH of the system exerts profound influence on the sorption of adsorbate molecule due to its influence on the surface properties of the adsorbent and ionization/dissociation of the adsorbate molecule. Figure 5.1 depicts the pH of highest sorption efficiency (pH 3), the percentage sorption increased from 70% at pH 2 to 81% at 3 and significantly decreases with increase of pH. The pH trend observed in this case is shown in Figure 5.1; from this study we can conclude that at pH 3 *Bacillus subtilis* showed maximum percent of biosorption.

5.1.2.2 Effect of biomass concentration

Biosorption of Chromium observed at various biomass concentrations of *Bacillus subtilis*. To achieve the maximum biosorption capacity of the biosorbent for Chromium, the biomass concentration was varied from 0.5 to 3 mg/ml. The variation in sorption from Figure 5.2 was observed; the optimum biomass concentration noted at 2 mg/ml and beyond this it is constant. This may be due to the unavailability of binding sites to the metal and also due to the blockage of binding sites with excess biomass.

5.1.2.3 Effect of temperature

Effect of temperature on Chromium biosorption is presented in Figure 5.3. Sorption experiments were conducted from10^{o}C to 50^{o}C. Good sorption percentages around 70% were observed in the temperature range of 24^{o}C to 40^{o}C. Maximum biosorption percentage of 81% was noted at 32^{o}C. In these experiments there was an increase in sorption percentage with increase in the temperature. A gradual decrease with further increase in temperature was noted. This is because of the shrinkage of cells in the higher and lower temperatures which will reduce the surface area of contact.

5.1.2.4 Effect of contact time

The sorption experiments of Chromium were carried out for different contact times with a fixed adsorbent dose of 2 mg/ml concentration at pH 3 at 32^{o}C. The results were plotted in Figure 5.4. The sorption percentage of metal increased with increase in contact time. The equilibrium time was 25 min for Chromium (81%).

5.1.3 Biosorption studies on Chromium using mixed cultures of Pseudomonas aeruginosa and Bacillus subtilis.

Present investigation deals with the utilization of mixed cultures of gram positive and gram negative bacteria as biosorbent for the sorption of Chromium. Considering the advantage of both bacterial surfaces in biosorption, studies on Chromium sorption using this mixed biomass were done. Batch studies were done to address various experimental parameters like pH, contact time, adsorbent dose for the sorption of Chromium. Greater percent of sorption was observed at lower concentrations of metal. Adsorption isotherms and kinetic studies were done. The effect of various parameters on the biosorption of

Chromium using mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) were discussed below.

5.1.3.1 Effect of pH

The sorption treatment of metals in water is pH dependent. However, pH is also known to affect the sorption process as magnitude of electrostatic charges imparted by ionized metal molecules is controlled by the pH of the medium. The percent of metal sorption vary with pH of the medium. The experimental results of Chromium sorption using mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) at varying pH ranges was shown in the Figure 5.1. Effect of pH on biosorption has been studied over a range of 1 to 7. Mixed cultures showed high sorption percent of around 77% at pH 3. Figure 5.1 shows a decrease in sorption percentage with further increase of pH.

5.1.3.2 Effect of biomass concentration

The effect of biomass concentration on sorption of Chromium was studied using various amounts ranging from 0.5 mg/ml to 3 mg/ml. These studies were done at a fixed pH 5. The Figure 5.2 depicts the effect of biomass concentration on sorption percentage of Chromium. It was observed the biosorption percentage increased with increase in biomass. Significantly high biosorption of around 77% was achieved at 1.5mg/ml and this biomass concentration was chosen for all further studies. It was observed that there is no further increase in the percentage sorption of Chromium with increase of biomass beyond 1.5 mg/ml. From this we can conclude that 1.5 mg/ml concentration of biomass gives optimum biosorption for Chromium with mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1).

5.1.3.3 Effect of temperature

Biosorption studies of Chromium using mixed cultures were carried out at different temperatures ranging from 10^{o}C to 50^{o}C. The effect of temperature on metal sorption is presented in Figure 5.3. The percentage of metal sorpted was increased from 10^{o}C to 32^{o}C and then showed decrease in sorption percentage with further increase in temperature. Figure 5.3 shows a maximum percent of sorption of around 77% was achieved at 32^{o}C at fixed pH and biomass concentrations.

5.1.3.4 Effect of contact time

Biosorption experiments of Chromium using mixed cultures were carried out for different contact times at fixed pH, biomass concentration and temperature. The Figure 5.4 depicts the percent sorption with time. The sorption percentage of the metal increased with time and a sorption of 77% was reached 25 min, The sorption of metal was rapid in the initial stages of contact time and gradually decreases with lapse of time until saturation. Maximum sorption was achived with *Bacillus subtilis* compared to *Pseudomonas* and mixed cultures.

5.1.3.5 Rate kinetics

Biosorption of metal onto biomass was monitored specific time intervals of 5min. the metal uptake was calculated from the data obtained from the metal uptake was plotted against time to determine a suitable kinetic model, the adsorption data was fitted into first order and second order kinetics (kinetic models desribed in Chapter 3) . The first order equation was plotted for ln (q_e-q_t) against t. the values of ln (q_e-q_t) were calculated from the kinetic data of Figure 5.6(a), 5.6(b). The k_1 values were calculated from the slope of this plot. The value of k_1 was shown in Table-5.2.

The second order equation was plotted for t/q_t against t. The values of q_e and k_2 are calculated from the slope and intercept of this plot. The values of q_e and k_2 are shown in Table-5.2. The correlation coefficient $R^2 = 0.984$ for pseudo-first order and $R^2 = 0.990$ for pseudo-second order kinetic equation states that both values are very near and well suited. But pseudo-second order best fitted with experimental values as it very near to 1.

By maintaining all the parameters at optimum levels the initial metal concentrations were varied (10, 15, 20, 25, 30 mg/L). The percentage sorption was decreased constantly with increase in initial metal concentration. The decline in the percentage biosorption was depicted in Figure 4.5.

5.1.3.6 Adsorption isotherms

The equilibrium experimental results of Chromium ions have been fitted in the Langmuir and Freundlich models. For biosorption of Chromium using mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) the coefficient of determination (R^2) of both models was mostly greater than 0.95 and close to 1(Figure 5.7(a), 5.7(b)). This

indicates that both models adequately describe the experimental data of the biosorption of Chromium.Data evaluation was done in the same manner as described in (chapter 3).

In the biosorption of Chromium by mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis*, most of the metal ions were sequestered very fast from the solutions in the first phase of contact time 30 minutes and almost no increase in the level of bound metal have been occurred after this time interval as shown in Figure 5.4. The sorption performance of the mixed biosorbent was studied under fixed environmental conditions. Biosorption equilibrium isotherms were plotted for metal uptake q against the residual metal concentration in the solution. The q verses C_f sorption isotherm relationship was mathematically expressed by Langmuir and Freundlich models. The higher the values of k and n; lower the value of b, the higher the affinity of the biomass. Table-5.3 describes summaries of linear regression data for Langmuir and Freundlich isotherms for Chromium biosorption using attenuate mixed biomass. Langmuir and Freundlich constant k were obtained from the linear equations of both models. As indicated in the Table-5.3, the coefficients of determination (R^2) of both models are 0.99 close to 1. In the Table-5.3 the values of K_f, 1/n, Q_{max} and b were given.

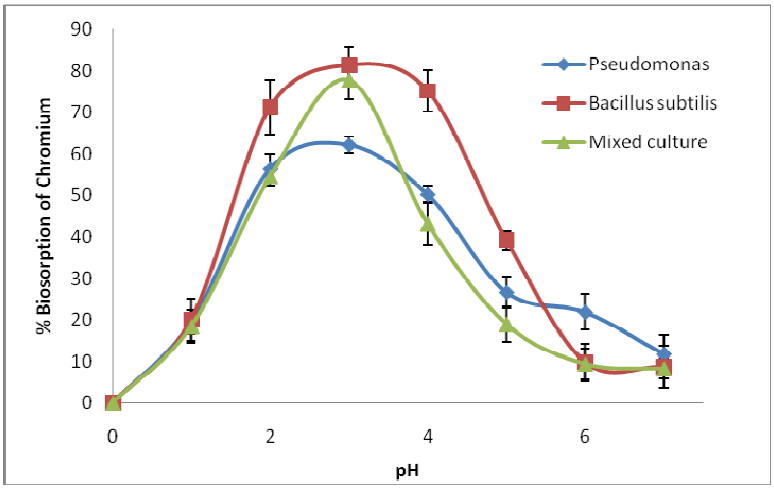

Figure 5.1: Effect of pH on percent Chromium biosorption at 32 ^oC temperature, (1.5, 2, 1.5 mg/ml) biomass, (30, 25, 25 min) of contact time and 10 mg/L initial metal concentration for *Pseudomonas aeruginosa, Bacillus subtilis* and mixed culture (1:1).

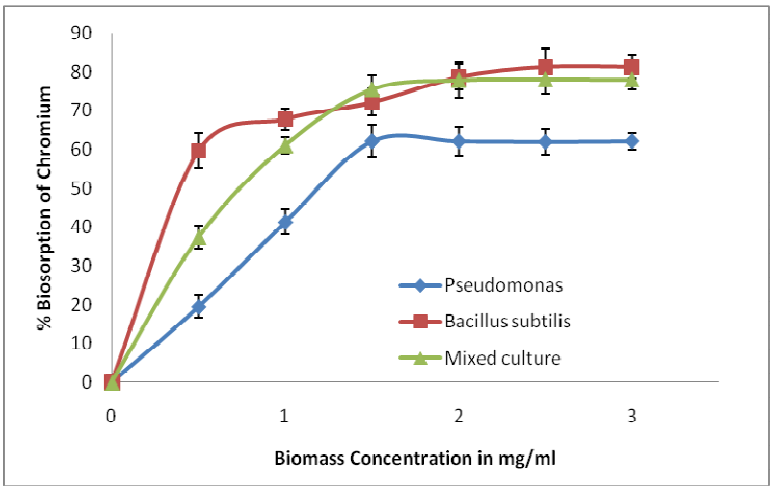

Figure 5.2: Effect of biomass concentration on percent Chromium biosorption at 32 ^{o}C temperature, pH 3, contact time (30, 25, 25 min) and 10 mg/L initial metal concentration for *Pseudomonas aeruginosa*, *Bacillus subtilis* and mixed culture (1:1).

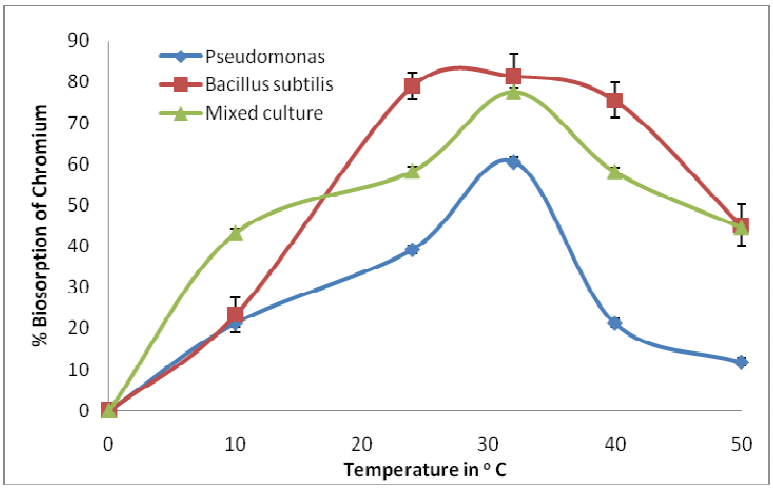

Figure 5.3: Effect of temperature on percent Chromium biosorption at pH 3, (1.5, 2, 1.5 mg/ml) biomass, contact time (30, 25, 25 min) and 10 mg/L initial metal concentration for *Pseudomonas aeruginosa*, *Bacillus subtilis* and mixed culture (1:1).

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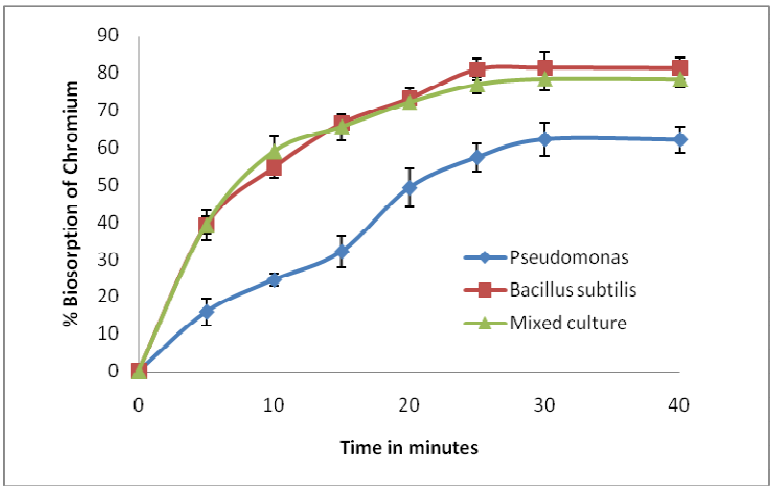

Figure 5.4: Effect of time on percent Chromium biosorption at 32 ^{o}C temperature, pH 3, (1.5, 2, 1.5 mg/ml) and 10 mg/L initial metal concentration for *Pseudomonas aeruginosa*, *Bacillus subtilis* and mixed culture (1:1).

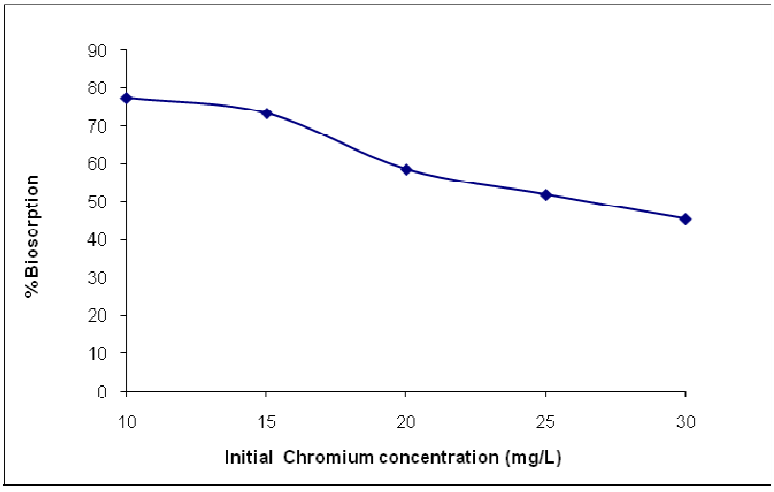

Figure 5.5: Effect of initial metal concentration at 1.5 mg/ml biomass, 32^{o}C temperature, pH 3, 30 min of contact time.

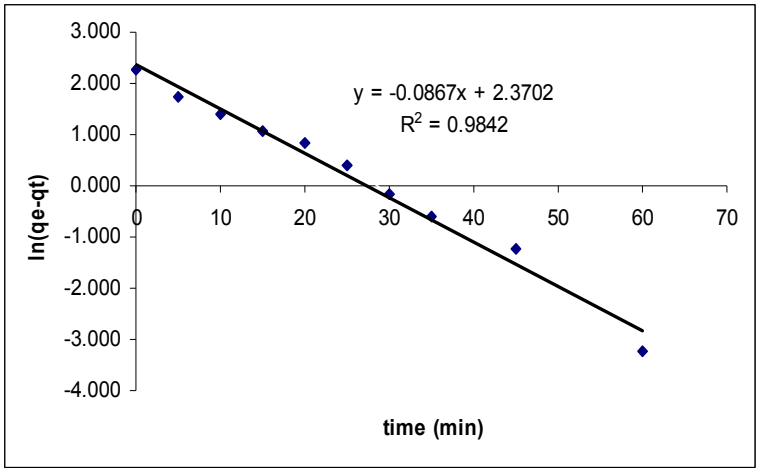

Figure 5.6(a): First order kinetics for Chromium by *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) mixed culture at 1.5 mg/ml biomass concnetration, 10 mg/L metal concentration, pH.3, 32^{o}C temperature.

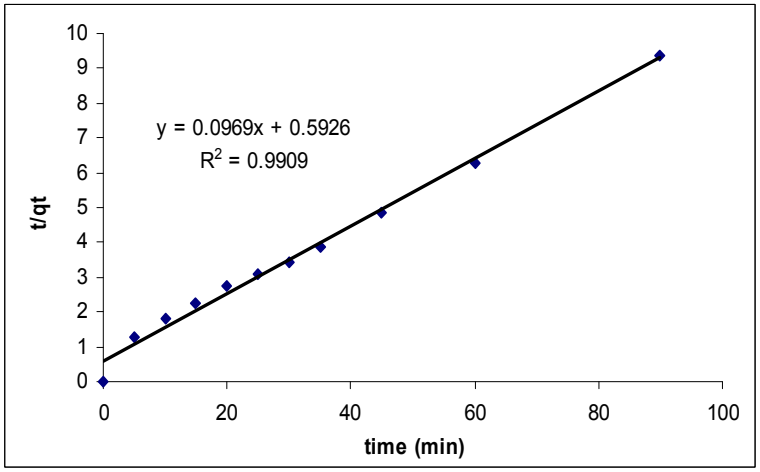

Figure 5.6(b): Second order kinetics for Chromium by *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) mixed culture at 1.5 mg/ml biomass concnetration, 10 mg/L metal concentration, pH.3, 32^{o}C temperature.

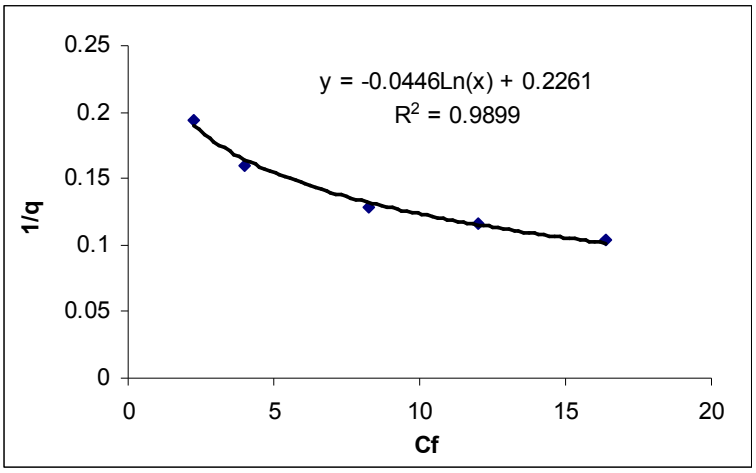

Figure 5.7(a): Adsopriton isotherm (Langmiur) for Chromium by *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) mixed culture at pH 3, 1.5 mg/ml biomass, 32^{o}C temperature, and 30 min of contact time.

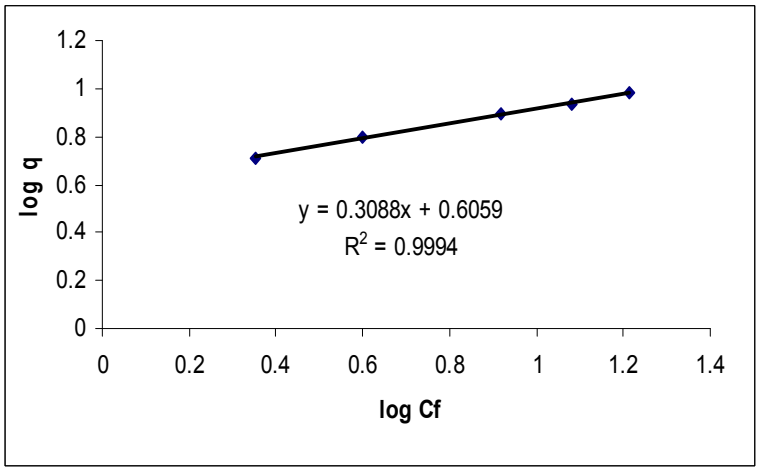

Figure 5.7(b): Adsopriton isotherm (Freundlich) for Chromium by *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) mixed culture at pH 3, 1.5 mg/ml biomass, 32^{o}C temperature, and 30 min of contact time.

Table-5.1: Kinetic data of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) mixed culture for Chromium.

Metal	Pseudo first order			Pseudo second order		
	K_1	q_e	R^2	K_2	q_e	R^2
Chromium	0.0867	10.69953	0.9842	0.1584	10.32	0.9909

Table-5.2: Parameters of isotherm models for heavy metal Chromium.

Metal	Freundlich parameters			Langmuir parameters		
	K_f	1/n	R^2	q_m	b	R^2
Chromium	4.0355	0.3088	0.994	4.422	-5.06	0.9899

Chapter 6

Biosorption of Arsenic using individual and mixed cultures of *Bacillus subtilis* and *Pseudomonas aeruginosa*

6.1 Results and Discussion

6.1.1 Biosorption studies using attenuated cells of Pseudomonas aeruginosa

Biosorption by *Pseudomonas aeruginosa* was performed by attenuated cell biomass with different parameters like pH, biomass concentration, temperature and time. The effect of each parameter is identified.

6.1.1.1 Effect of pH

pH is the most important factor influencing the sorption efficiency. Both cation and anion show a different pattern of sorption on sorbent in the same pH range. From Figure 6.1, it is revealed that maximum sorption in percent achieved at pH 5. The sorption percentage was increased with increase in pH and reached a maximum at pH 5; beyond it followed a decrease with increase in pH. When compared to the sorption studies of Mercury, Chromium as explained in Chapters 4, 5; Arsenic sorption is low by *Pseudomonas* culture. This may be due to less affinity of the cells to Arsenic metal ions.

6.1.1.2 Effect of biomass concentration

Batch experiments were conducted to investigate the influence of biomass concentration on the percentage sorption of Arsenic. To achieve the maximum biosorption capacity of the biosorbent for Arsenic, the biomass concentration was varied from 0.5 to 5 mg/ml and it was found that a concentration of 3 mg/ml was sufficient for maximum percentage of Arsenic biosorption under the reported experimental conditions. These finding are shown in Figure 6.2. It is also seen from this Figure that a further increase in biomass does not affect the sorption percentage greatly. This may be due to the unavailability of binding sites to the metal and also due to the blockage of binding sites with excess biomass.

6.1.1.3 Effect of temperature

Effect of temperature on Arsenic biosorption is presented in Figure 6.3. With increase in temperature the Arsenic sorption increased and showed a maximum at 32^{o}C. It was noticed that further increase in temperature resulted in a decrease of sorption capacity. The Arsenic sorption capacity was low and the decrease may be due to shrinkage of cells in the higher and lower temperatures which reduces the surface area of contact.

6.1.1.4 Effect of contact time

The adsorption experiment of Arsenic was carried out for different contact times with a fixed adsorbent dose of 1.5 mg/ml concentration at pH 5 at 32^{o}C. The results were plotted in Figure 6.4. The sorption percentage of metal increased with increase in contact time. The equilibrium time was 15 min for Arsenic (30.4%).

6.1.2 Biosorption studies using attenuated Bacillus subtilis

In the studies carried out so far, the attenuated cells of *Bacillus subtilis* were used for the biosorption of Arsenic. The parameters affecting the Biosorption of Arsenic using this single culture of *Bacillus subtilis* were studied. The effect of these parameters was discussed below.

6.1.2.1 Effect of pH

The pH of the system exerts profound influence on the sorption of adsorbate molecule due to its influence on the surface properties of the adsorbent and ionization/dissociation of the adsorbate molecule. Figure 6.1 depicts the percentage sorption increased with pH and highest sorption efficiency obsderved at pH 6; beyond this point subsequent decreases is seen with increase of pH. From this study we can conclude that optimum pH for *Bacillus subtilis* for biosorption is 6 and 28.67% sorption observed.

6.1.2.2 Effect of biomass concentration

Biosorption of Arsenic in percentage observed to various biomass concentrations of *Bacillus subtilis*. To achieve the maximum biosorption capacity of the biosorbent for Arsenic, the biomass concentration was varied from 0.5 to 5 mg/ml. The variation in sorption from Figure 6.2 was observed; the optimum biomass concentration noted at 3

mg/ml (30.46% biosorption) and beyond it is constant. This may be due to the unavailability of binding sites to the metal and also due to the blockage of binding sites with excess biomass.

6.1.2.3 Effect of temperature

Effect of temperature on Arsenic biosorption is presented in Figure 6.3. Sorption experiments were conducted from10^{o}C to 50^{o}C. Good sorption percentage of around 28.6% was observed at the temperature 32^{o}C. With increase in temperature the percentage sorption increased and begins to reduce at beyond 32^{o}C. This is because of the shrinkage of cells in the higher and lower temperatures which will reduce the surface area of contact. The Arsenic sorption by *Bacillus* observed to be lower than other cultures used in the experiment (Figure 6.3).

6.1.2.4 Effect of contact time

The sorption experiments of Arsenic were carried out for different contact times with a fixed adsorbent dose of 2 mg/ml concentration at pH 3 at 32^{o}C. The results were plotted in Figure 6.4. The sorption percentage of metal increased with increase in contact time. The equilibrium time was 20 min for Arsenic (28.61% biosorption).

6.1.3 Biosorption studies on Arsenic using mixed cultures of Pseudomonas aeruginosa and Bacillus subtilis.

Present investigation deals with the utilization of mixed cultures of gram positive and gram negative bacteria as biosorbent for the sorption of Arsenic. Considering the advantage of both bacterial surfaces in biosorption, studies on Arsenic sorption using this mixed biomass were done. Batch studies were done to address various experimental parameters like pH, contact time, adsorbent dose for the sorption of Arsenic. Greater percent of sorption was observed at lower concentrations of metal. Adsorption isotherms and kinetic studies were done. The effect of various parameters on the biosorption of Arsenic using mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) were discussed below.

6.1.3.1 Effect of pH

The sorption treatment of metals in water is pH dependent. However, pH is also known to affect the sorption process as magnitude of electrostatic charges imparted by

ionized metal molecules is controlled by the pH of the medium. The percent of metal sorption vary with pH of the medium. The experimental results of Arsenic sorption using mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) at varying pH ranges was shown in the Figure 6.1. Effect of pH on biosorption has been studied over a range of 1 to 7. Mixed cultures showed high sorption percent of around 30.47% at pH 5. Figure 6.1 shows a decrease in sorption percentage with further increase of pH.

6.1.3.2 Effect of biomass concentration

The effect of biomass concentration on sorption of Arsenic was studied using various amounts ranging from 0.5 mg/ml to 5 mg/ml. These studies were done at a fixed pH 5. The Figure 6.2 depicts the effect of biomass concentration on sorption percentage of Arsenic. It was observed the biosorption percentage increased with increase in biomass. Significantly high biosorption around 30.46% was achieved at 3 mg/ml and this biomass concentration was chosen for all further studies. It was observed that there is no further increase in the percentage sorption of Arsenic with increase of biomass beyond 3 mg/ml. From this we can conclude that 3 mg/ml concentration of biomass gives optimum biosorption for Arsenic with mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) which was a better result than using *Bacillus subtilis* alone (Figure 6.2).

6.1.3.3 Effect of temperature

Biosorption studies of Arsenic using mixed cultures were carried out at different temperatures ranging from 10^{o}C to 50^{o}C. The effect of temperature on metal sorption was presented in Figure 6.3. The percentage of metal sorpted was increased from 10^{o}C to 32^{o}C and then showed decrease in sorption percentage with further increase in temperature. Figure 6.3 shows a maximum percent of sorption of around 30.4% was achieved at 32^{o}C at fixed pH and biomass concentration.

6.1.3.4 Effect of contact time

Biosorption experiments of Arsenic using mixed cultures were carried out for different contact times at fixed pH, biomass concentration and temperature. The Figure 6.4 depicts the percent sorption with time. The sorption percentage of the metal increased with time and a sorption of 30.47% was reached at 15 min and remained almost constant with increase upto 40 min. The sorption of metal was rapid in the initial stages of contact time and gradually decreases with lapse of time until saturation. From the Figure 6.4 the

sorption property with time for mixed cultures stood in between the *Bacillus subtilis* and *Pseudomonas*. Effect of contact time for biosorption of Arsenic was investigated at fixed tempareture, biomass and pH.

6.1.3.5 Rate kinetics

Biosorption of metal onto biomass was monitored specific time intervals of 5min. the metal uptake was calculated from the data obtained from the metal uptake was plotted against time to determine a suitable kinetic model (shown in Chapter 3), the adsorption data was fitted into first order and second order kinetics. The first order equation was plotted for ln (q_e-q_t) against t. the values of ln (q_e-q_t) were calculated from the kinetic data of Figure 6.6(a), 6.6(b). The k_1 values were calculated from the slope of this plot. The value of k_1 was shown in Table-6.1.

The second order equation was plotted for t/q_t against t. The values of q_e and k_2 are calculated from the slop and intercept of this plot. The values of q_e and k_2 are shown in Table-6.1. The correlation coefficient $R^2 = 0.825$ for pseudo-first order and $R^2 = 0.964$ for pseudo-second order kinetic equation states that pseudo-second order best fitted with experimental values as it close to 1.

By maintaining all the parameters at optimum levels the initial metal concentrations were varied (5, 10, 15, 20, 25 mg/L). There is steep decline in percentage sorption from 5 mg/L to 10 mg/L and then decreased constantly with increase in initial metal concentration. The decline in the percentage biosorption was depicted in Figure 4.5.

6.1.3.6 Adsorption isotherms

The equilibrium experimental results of Arsenic ions have been fitted in the Langmuir and Freundlich models. For biosorption of Arsenic using mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) the coefficient of determination (R^2) of both models was mostly greater than 0.95 and close to 1(Figure 6.7(a), 6.7(b)). This indicates that both models adequately describe the experimental data of the biosorption of Arsenic. Data evaluation was done in the same manner as described in (chapter 3).

In the biosorption of Arsenic by mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1), most of the metal ions were sequestered very fast from the solutions in the first phase of contact time 15 minutes and almost no increase in the level

of bound metal have been occurred after this time interval as shown in Figure 6.4. The sorption performance of the mixed biosorbent was studied under fixed environmental conditions. Biosorption equilibrium isotherms were plotted for metal uptake q against the residual metal concentration in the solution. The q verses C_f sorption isotherm relationship was mathematically expressed by Langmuir and Freundlich models. The higher the values of k and n; lower the value of b, the higher the affinity of the biomass. Table-6.2 describes summaries of linear regression data for Langmuir and Freundlich isotherms for Arsenic biosorption using attenuate mixed biomass. Langmuir and Freundlich constant k were obtained from the linear equations of both models. As indicated in the Table-6.2, the coefficients of determination (R^2) of both models are 0.99 close to 1. In the Table-6.2 the values of K_f, 1/n, Q_{max} and b were given.

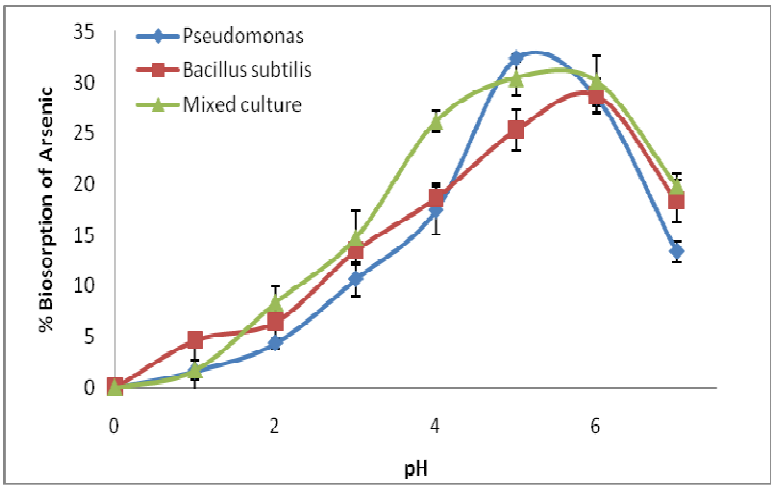

Figure 6.1: Effect of pH on percent Arsenic biosorption at 32 ^oC temperature, 3 mg/ml biomass concentration, (15, 20, 15 min) of contact time and 10 mg/L initial metal concentration for *Pseudomonas aeruginosa*, *Bacillus subtilis* and mixed culture (1:1).

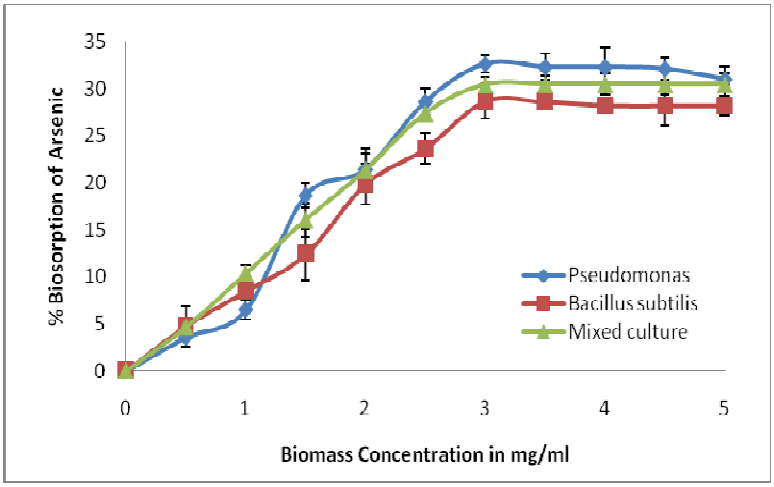

Figure 6.2: Effect of biomass concentration on percent Arsenic biosorption at 32 ^{o}C temperature, pH (5, 5, 6), contact time (15, 20, 15 min) and 10 mg/L initial metal concentration for *Pseudomonas aeruginosa*, *Bacillus subtilis* and mixed culture (1:1).

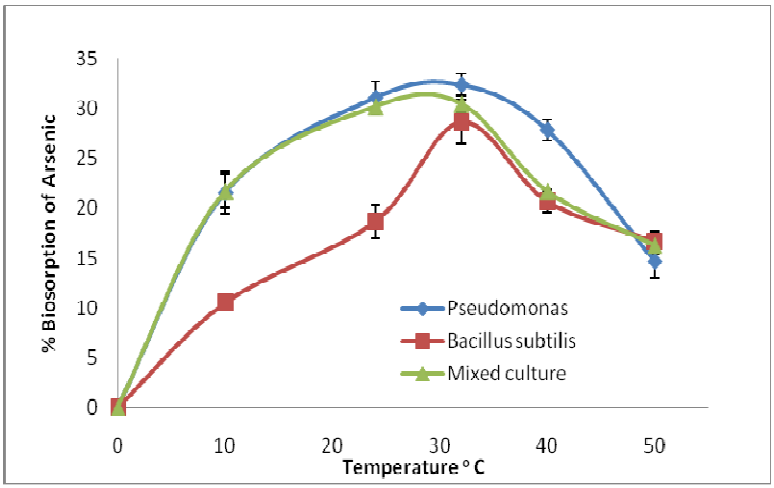

Figure 6.3: Effect of temperature on percent Arsenic biosorption at pH (5, 5, 6), 3 mg/ml biomass, contact time (15, 20, 15 min) and 10 mg/L initial metal concentration for *Pseudomonas aeruginosa*, *Bacillus subtilis* and mixed culture (1:1).

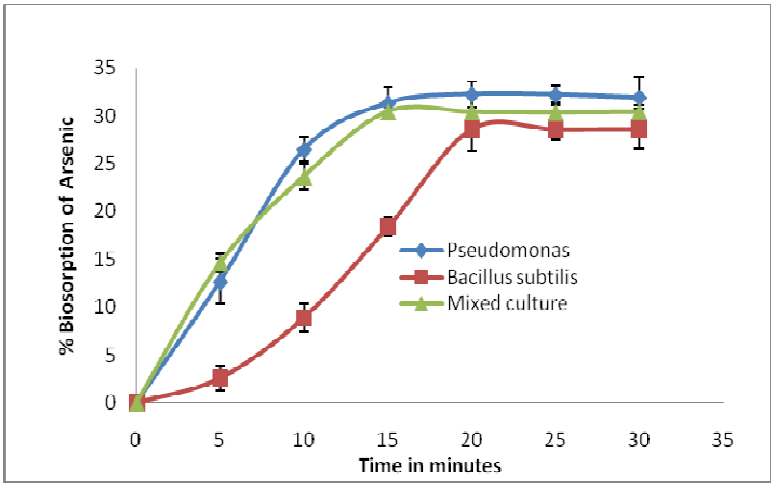

Figure 6.4: Effect of time on percent Arsenic biosorption at 32 ^{o}C temperature, pH (5, 5, 6), for *Pseudomonas aeruginosa*, *Bacillus subtilis* and mixed culture at 3 mg/ml biomass concentration (1:1).

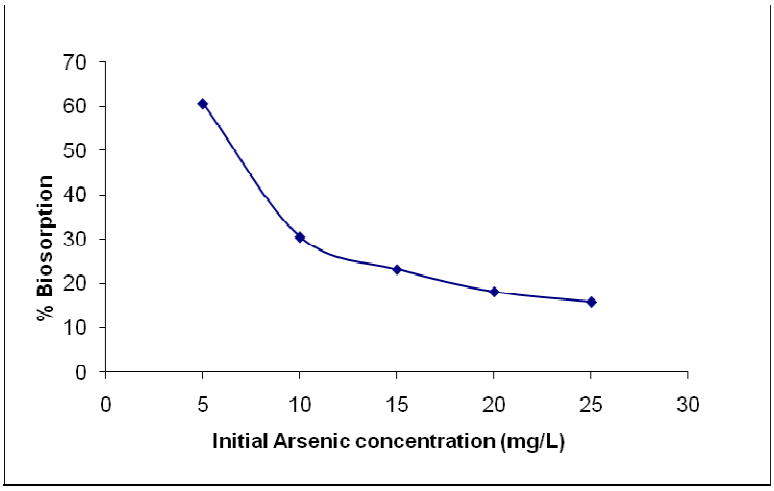

Figure 6.5: Effect of initial metal concentration at 3 mg/ml biomass, 32^{o}C temperature, pH 6, 15 min of contact time.

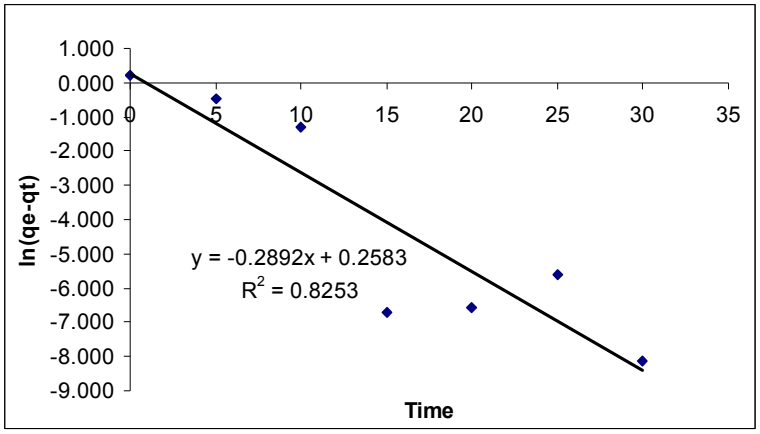

Figure 6.6(a): First order kinetics for Arsenic by *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) mixed culture at 3 mg/ml biomass concnetration, 10 mg/L metal concentration, pH.6, 32^{o}c temperature.

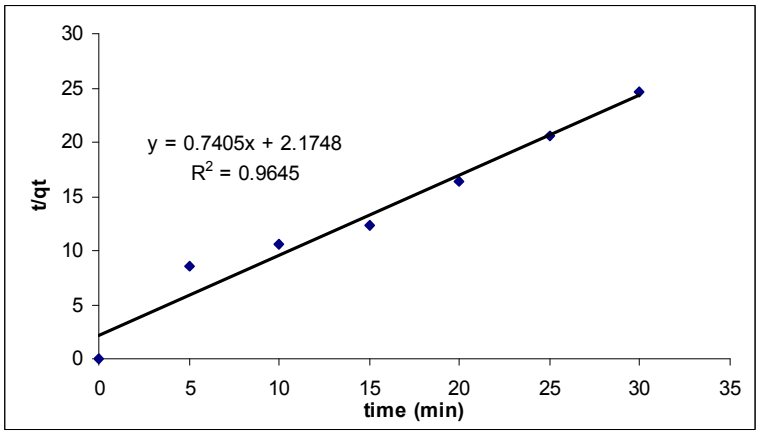

Figure 6.6(b): Second order kinetics for Arsenic by *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) mixed culture at 3 mg/ml biomass concnetration, 10 mg/L metal concentration, pH.6, 32^{o}c temperature.

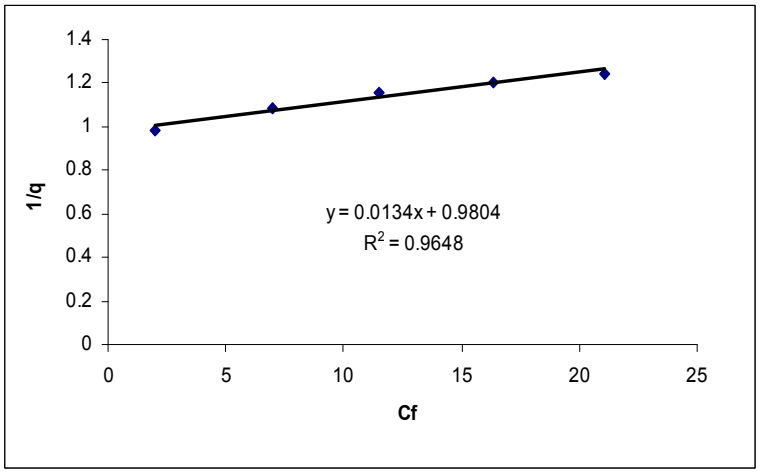

Figure 6.7(a): Adsopriton isotherm (Langmiur) for Arsenic by *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) mixed culture at pH 6, 3 mg/ml biomass, 32^{o}C temperature, 15 min of contact time.

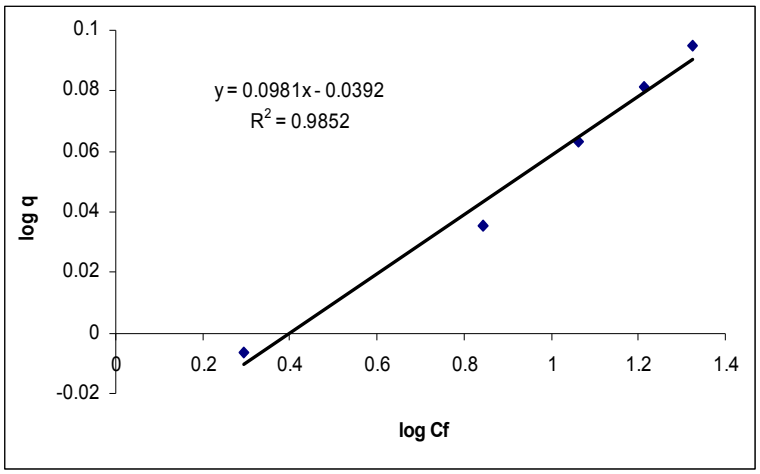

Figure 6.7(b): Adsopriton isotherm (Freundlich) for Arsenic by *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) mixed culture at pH 6, 3 mg/ml biomass, 32^{o}C temperature, 15 min of contact time.

Table-6.1: Kinetic data of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) mixed culture for Arsenic.

Metal	Pseudo first order			Pseudo second order		
	K_1	q_e	R^2	K_2	q_e	R^2
Arsenic	0.2892	1.294727	0.8253	0.252	1.3504	0.9645

Table-6.2: Parameters of isotherm models for heavy metal Arsenic.

Metal	Freundlich parameters			Langmuir parameters		
	K_f	1/n	R^2	q_m	b	R^2
Arsenic	1.0944	0.0981	0.9852	1.0199	73.529	0.9648

Chapter 7

Biosorption of binary mixtures of heavy metal solutions using mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis.*

7.1 Introduction

Although actual wastewater treatment systems often have to deal with a mixture of heavy metals, most research work still only focuses on a single metal sorption. Only a few works on the sorption of mixture of heavy metals were found in literature. For instance, Sag and kustal (1995) investigated the competitive biosorption of Cr (6+) and Fe (3+) by *Rhizopus arrhizus* and reported that instantaneous, equilibrium and maximum uptake of Cr (6+) and Fe (3+) were reduced by increasing concentration of other metals. The combined action of competitive uptake of these two was generally found to be antagonist. The most logical reason for the antagonist action was claimed to be the competition for sorption site on the cell and/ or screening effect by the second metal. In the examination of the biosorption of Cu 2+ and Zn 2+ by *Cymodocea nodosa*, (sanchez et al. (1999)) stated that there was a competition between the two metal species and Cu 2+ was preferentially adsorbed by this biomass kaewsam (2000) investigated the effect of other heavy metals ions on Cu 2+ uptake by *Durvilaes potatorum*. It was reported that Cu 2+ uptake was significantly affected by other heavy metals (Ag+, Mn2+, Co2+, Ni2+, Fe2+, Cd2+, Pb2+) and EDTA because the metal binding sites on the biosorbent were limited, so these ions competed simultaneously for the site. The amount of suppression for Cu2+ depended on the affinity of these ions for binding strength of the respective heavy metal ions to the biosorbent. Singh et al. (2001) studied the multi-metals combination between Ni2+ and Cr6+ by *Microcystis sp*. They found that Ni2+ sorption capacity by this biomass was higher than that of Cr6+ as the binding sites in the biomass had a greater affinity for Ni2+.

The purpose of present work is to study the mixed culture capabilities of *Pseudomonas* and *Bacillus* in sorption of binary mixtures of heavy metals. Here we used the optimized parameters obtained from our study described in chapters 5, 6, 7.

7.2 Experimental setup

Biosorption experiments were carried out in 250 ml conical flasks using 100 ml metal solution with required amount of biosorbent. The biomass of mixed cultures was prepared accordingly as described in the chapter 3. Here the biomass of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) were mixed in1:1 ratio. The metal solution was also prepared in the same way as described in the chapter 3. Here the metal solutions were mixed in equal amounts. For conducting the experiments pH was adjusted and flasks were continuously shaken for required time period. The samples were collected at regular time intervals and analysed**.**

7.3 Results and discussion

7.3.1 Biosorption of Mercury-Chromium binary solution

The Mercury-Chromium binary mixture was prepared by mixing the metal solutions in equal amounts. Here in these experiments Mercury and Chromium concentrations were 10mg/L each. Mixed biomass is also prepared by adding the individual biomass in equal amounts. Here in this experiment *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) biomass concentrations were 1mg/ml each. The temperature was set at 32^{o}C, which is optimum temperature in case of Mercury and Chromium biosorption with the same biomass. The pH was set at 4 as it is intermediate between the optimum pH of Chromium (pH 3) and Mercury (pH 5) by mixed culture. The Figure 7.1 depicts the sorption percentage both the metals with mixed culture. Here a maximum sorption of 74% and 30% for Chromium and Mercury were observed. The equilibrium time for Chromium sorption was found to be 50 minutes and the equilibrium time for Mercury sorption was found to be 40 minutes. Mercury showed a percentage sorption of 90% (Figure 7.1) in single metal solution system but showed only 30% sorption percentage in binary metal solutions. This indicated that there was a stronger competitive sorption took place in case of binary solutions.

7.3.2 Biosorption of Mercury and Arsenic binary solution

The Mercury-Arsenic binary mixture was prepared in the same manner as explained in the above section of Mercury-Chromium binary solution. The biomass concentration of 3 mm/ml was taken i.e.; 1.5 mg/ml concentration of each individual biomass. The experiments were conducted at 32^{o}C which is the optimum temperature for both the metals which was found from the previous chapters 5 and 6. Here the pH was adjusted at 5 which is the optimum pH for both Mercury and Arsenic with mixed cultures. The Figure 7.2 presents the biosorption percentages of Mercury and Arsenic in binary solutions. Here from the Figure it is evident that in binary solutions the maximum sorption of Mercury is 70.7 percent and for Arsenic it is 20.9 percent. Arsenic sorption was low due to the presence of negatively charged oxyanion, which may relate with repulse electrostactic interactions between negatively charged surface of biomass and Aso_4^{3-}. Arsenic biosorption was rapid and attained equilibrium with in 15 minutes. This may be due to the rapid occupation of available positive site on the cell surface. Mercury sorption reached the equilibrium at 50 minutes. A sorption percent of 70 was observed for Mercury.

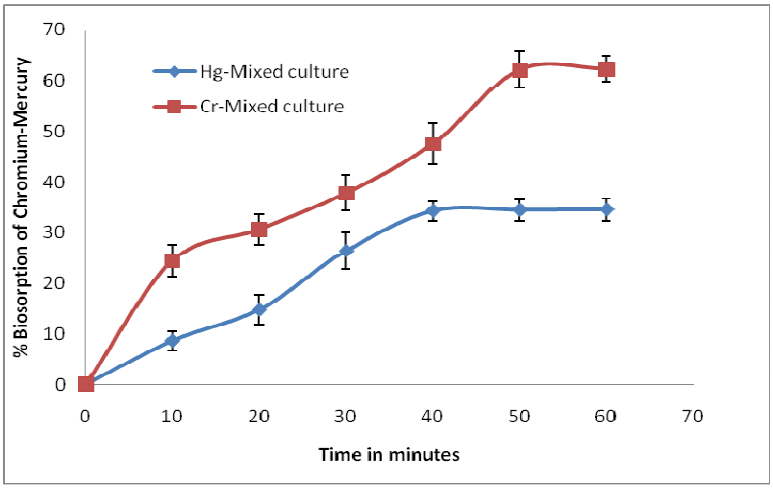

Figure 7.1: Biosorption of binary metal mixture of Chromium-Mercury by mixed culture of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) at 10ml/L each, 1 mg/ml biomass concnetration, pH.4 and 32^{o}c temperature.

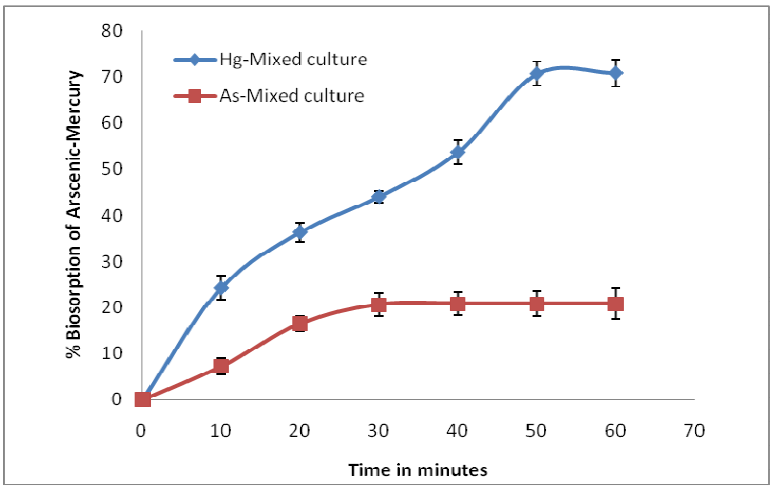

Figure 7.2: Biosorption of binary metal mixture of Arsenic-Mercury by mixed culture of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1) at 10ml/L each, 3 mg/ml biomass concnetration, pH.5 and 32^{o}c temperature.

Chapter 8

Summary and Conclusions

Biosorption of three heavy metals namely Mercury, Chromium and Arsenic were conducted using individual and mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1). The Mercury biosorption studies using individual cultures of *Pseudomonas aeruginosa* resulted 99.3% of sorption at pH 5, temperature 32^{o}C and biomass concentration of 0.5 mg/ml in 50 minutes period of contact time. Mercury biosorption studies were also performed using individual cultures of *Bacillus subtilis* and it was found that 78.5% Mercury removal at pH 5, temperature 32^{o}C and biomass concentration of 2.5 mg/ml in 60 minutes period of contact time. For mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1), studies showed a sorption of 90% for Hg and optimum pH was again found to be pH 5 for the biomass concentration of 2 mg/ml at the temperature of 32^{o}C. From these studies it was observed that the sorption capacity of *Pseudomonas aeruginosa* is far higher when compared with mixed cultures and *Bacillus subtilis*.

Further investigations were carried out for Chromium using individual and mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis* (1:1). Results showed that maximum sorption of Chromium were observed at 3 pH. A maximum sorption of 60.5% was observed using *Pseudomonas aeruginosa* at the optimum dosage and temperature of 1.5 mg/ml at 32^{o}C respectively. Similar investigations were conducted for Chromium using *Bacillus subtilis.* A maximum sorption of 81.3% was obtained for Chromium with an optimum biomass concentration of 2mg/ml, temperature 32^{o}C and pH-3. Biosorption of Chromium using mixed culture was explored in the present work and 77.6% sorption was observed. The optimum conditions for this sorption were 1.5 mg/ml biomass, pH 3 and temperature 32^{o}C. From this biosorprion study, it can be concluded that among the three systems *Bacillus subtilis* showed very good sorption capacity for Chromium biosorption.

In Arsenic biosorption studies using individual cultures of *Pseudomonas aeruginosa* a meager sorption of 32% was observed at pH 5, biomass 3 mg/ml and temperature 32^{o}C in 15 minutes. Biosorption studies on Arsenic using individual culture of *Bacillus subtilis* revealed even lesser sorption of 28% at pH-6, biomass 3 mg/ml and temperature 32^{o}C in 15 minutes. Similar studies for Arsenic with mixed culture resulted in a sorption of 30 percent at pH 5, 3 mg/ml biomass and 15 minutes contact time. From these studies of Arsenic biosorption it can be concluded that very little percent of sorption was obtained for Arsenic due to the negative charged oxyanion, that may create a repulsive electrostatic interactions between negatively charged surface of biomass and AsO_4^{3-}. Here the negatively charged AsO_4^{3-} occupies the positive binding sites on the bacterial surfaces by stopping and further sorption will not take place. Hence Arsenic biosorption was very rapid when compared with other metals. The biosorption of binary metal solutions like Mercury-Chromium and Mercury-Arsenic using mixed culture were also tested. The maximum sorption of 74% and 30% for Chromium and Mercury respectively was observed at pH 4, temperature 32^{o}C and 2mg/ml biomass concentration. For Mercury-Arsenic binary system biosorption of Mercury:Arsenic was found to be 70.7%:20.9% at pH 5, temperature 32^{o}C and biomass concentration of 3mg/ml. From these studies it can be concluded that mixed cultures can be applied in binary metal sorption systems by taking the advantage of microbe's specificity in metal sorption. Isotherms namely Langmuir and Freundlich for all the three metals with mixed cultures were tested for maximum regression coefficient (R^2 =0.99). Two kinetic models pseudo first order equation and pseudo second order equation were used for the study of process kinetics. This sorption process was rapid in the initial stages and gradually there was a decrease in the sorption rate of heavy metals and later it became static. Thus an exhaustive study of biosorption of heavy metals using individual and mixed culture was performed optimizing various key process parameters for a maximum sorption.

Cell walls of bacteria are principally composed of peptidoglycans which consist of linear chains of the disaccharide *N*-acetylglucosamine-β 1,4-*N*acetylmuramic acid with peptide chains. Cell walls of gram negative bacteria are somewhat thinner than the gram positive ones and are also not heavily cross-linked. They have an outer membrane which is composed of an outer layer of lipopolysaccharide (LPS), phospholipids and proteins

(Remacle 1990). Biosorption mainly involves a) cell surface complexation, b) ion exchange or affinity and c) micro precipitation. Different microbes have been found to vary in their affinity for different heavy metal(s) and hence differ in their metal binding capacities. Some biomass (es) exhibit preference for certain heavy metal(s) whereas others do not show any specific binding and are broad range (Gupta, et al., 2000).

In our study on Chromium, *Bacillus subtilis* showed higher sorption than *Pseudomonas aeruginosa* and mixed culture. The affinity of Chromium towards gram positive *Bacillus subtilis* is because techoic acid was the prime binding site for the metal (cation) (Hoyle and Beveridge, 1983). Among the mechanisms ion affinity dominated due to higher charge density of Chromium (VI).

When it comes to Mercury the affinity was towards gram negative *Pseudomonas aeruginosa*. Here the major role is played by cell surface complexation mechanism than ion exchange or affinity. This may be due to the lower charge density of Mercury when compared to Chromium. Gram positve bacteria normally show low levels of surface complexation due to heavily cross linked peptidoglycan layer (Gupta, et al., 2000). Where as in gram negaitve bacteria most of their lipopolysaccharide (LPS), phospholipids and proteins are exposed on the cell surface and involves in cell surface complexation.

In case of Arsenic; biosorption was more for *P.aeruginosa* and is much lower in sorption percentage when compared to other two metals. The lower percentage in sorption is due to negative charge (Čerňanský et al., 2007). It is showing its affinity towards gram negative *P. aeruginosa* because the surface of gram positive *B.subtilis* was containing higher levels of carboxylic groups which repel this anion.

While coming to binary metal solutions with mixed cultures of *P.aeruginosa* and *B.subtilis*, all results are following the above phenomenon. In Mercury-Chromium binary solutions, Chromium was more sorpted and in Mercury-Arsenic binary solution Arsenic was more sorpted. The finding of this theis is biosorption of mercury using P. *aeruginosa*. Almost 100 percent metal is biosorpted to the biomass in optimum conditions. The effect of biomass dose on initial metal concentration need to be .studied further in detail for Chromium and Arsenic. This study may help enhancing their biosorption extent.

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Contributions

- ***Communicated***
  - Statistical optimization of process parameters for Cr(VI) biosorption onto mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis*, *Bioresource Technolgy.*
  - Communicated a paper on Heavy metal Biosorption using Attenuated cells of *Bacillus subtilis* and *Pseudomonas aeruginosa* in *Journal of environmental research and development* (JERAD), 2008.
- ***Conferences***
  - A paper on optimization of Heavy metal Biosorption using Attenuated cells of *Bacillus subtilis* and *Pseudomonas aeruginosa* was selected for the International Congress on Environmental Research and development (ICER-200), December 2008.