2.1. Environmental/Heavy metal pollution

Pollution can be defined as the presence in or introduction into the environment of a substance which has harmful or toxic effects that can cause disorder, and will be a nuisance to the biotic and abiotic system (Duruibe et al., 2007). Environmental pollution due to industrial urbanization leads to accumulation of large quantity of various pollutants including heavy metals in to biosphere (Filazi et al., 2003). Dumping of heavy metals in the geo-sphere of earth should be avoided because they are not eco-friendly. Due to the uncontrolled population, vast use of heavy metals has been increasing every day and their improper treatment in industries magnified this problem many folds. Chemical properties of the metals such as high dispersion, solubility, mobility, stability, anthropogenic activity, industrial and chemical process increases the transport of metals to the aquatic system (Carrillo-Gonzáles et al., 2006).

Prolonged exposure to the heavy metals at higher concentrations can cause many ailments such as skin lesions, growth retardation, lung infections, improper vision, congenital defects and teratogenic effects (Hughes and Poole, 1989; Poole and Gadd, 1989). Besides, aquatic organisms such as phytoplankton, zooplankton, fishes and even humans are severely affected by the heavy metals through biomagnifications and bioaccumulation process (Khayatzadeh et al., 2010). Heavy metals in higher quantities have been toxic and cause many diseases to all forms of living organisms and many researchers tried numerous varied approaches to remove or reduce the metal toxicity.
2.2. Significance of Heavy metals

Metals having density more than 5 gm/cm$^3$ are referred as heavy metals (Weast, 1984). In the modern era due to the unmindful urbanization, heavy metal concentration is increasing in the environment resulting in severe effects to both biotic and abiotic systems. In biological systems, heavy metals are referred to as trace elements since they are the part of biochemical reactions and need in nanomolar quantities. Metals like Co, Cu, Cr, Fe, Mg, Mn Mo, Ni, Si, and Zn are vital micronutrients and are necessary for various biochemical and physiological functions (Hamid et al., 2010). Despite their significant role in biological system, trace elements like zinc, nickel, cobalt and copper at higher concentrations, are known to be highly toxic to living beings (Omenn et al., 1995).

2.3. Toxicity of Heavy metals

Even though some heavy metals at lower concentration are very essential for life, most can be hazardous to all forms of life including microbes, humans, plants and animals. Heavy metals show their toxic effects by blocking the functional groups and altering the 3D structure of the proteins (Duruibe et al., 2007). Effects and toxicity of some of the heavy metals are listed below (Table- 2.1). Among the various heavy metals reported, cobalt is most significant since it is extensively used and easily dispersed in the environment due to the anthropogenic activities. Main sources of the cobalt contamination are coming from mining and processing industries.

Table 2.1: Effect and Toxicity of Heavy metals

<table>
<thead>
<tr>
<th>Metal</th>
<th>Toxicity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pb</td>
<td>Neurotoxic</td>
<td>Battery, architecture metal, oil paints, gasoline</td>
</tr>
<tr>
<td>Hg</td>
<td>Poisonous</td>
<td>Electrical and electronic applications</td>
</tr>
<tr>
<td>-----</td>
<td>---------------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>Cd</td>
<td>Carcinogenic, poisonous</td>
<td>Batteries and electroplating</td>
</tr>
<tr>
<td></td>
<td>neumonitis, Pulmonary edema</td>
<td></td>
</tr>
<tr>
<td>Co</td>
<td>Contact dermatitis</td>
<td>Batteries, catalysis, electroplating, Nuclear industries</td>
</tr>
<tr>
<td>Ni</td>
<td>Alloy metal rechargeable batteries</td>
<td>Batteries, electroplating,</td>
</tr>
<tr>
<td>Cu</td>
<td>Electrical motors, metal alloy</td>
<td>Catalysis, cable industries,</td>
</tr>
<tr>
<td></td>
<td>Architecture and industries</td>
<td></td>
</tr>
<tr>
<td>As</td>
<td>Arsenicosis</td>
<td>Wood preservative, military, car batteries and pesticides</td>
</tr>
</tbody>
</table>

2.4. Focus on cobalt

2.4.1. General properties of cobalt

Cobalt is one of the most important heavy metals having intense applications. The general properties of cobalt are listed below in the Table- 2.2.

Table 2.2: General properties of Cobalt

<table>
<thead>
<tr>
<th>Name and Symbol</th>
<th>Cobalt: Co</th>
</tr>
</thead>
<tbody>
<tr>
<td>State</td>
<td>Solid</td>
</tr>
<tr>
<td>Atomic Number</td>
<td>27</td>
</tr>
<tr>
<td>Element category</td>
<td>Transition Metal</td>
</tr>
<tr>
<td>Group: Period: Block</td>
<td>9: 4: d</td>
</tr>
<tr>
<td>Standard atomic weight</td>
<td>58.933195(5) g·mol⁻¹</td>
</tr>
<tr>
<td>Crystal structure</td>
<td>Hexagonal</td>
</tr>
<tr>
<td>Density</td>
<td>8.90 g·cm⁻³</td>
</tr>
<tr>
<td>Melting point</td>
<td>1495 ºC</td>
</tr>
<tr>
<td>Boiling point</td>
<td>2927 ºC</td>
</tr>
<tr>
<td>Magnetic ordering</td>
<td>Ferromagnetic</td>
</tr>
<tr>
<td>Electrical resistivity</td>
<td>(20 ºC) 62.4 nΩ·m</td>
</tr>
<tr>
<td>Thermal conductivity</td>
<td>100 W·m⁻¹·K⁻¹</td>
</tr>
</tbody>
</table>

2.4.2. Cobalt in Nature

Average concentration of cobalt reported was 23 ppm in the environment;
however, it is not uniform in all parts of the Earth. Concentration of cobalt in soil is varying according to the type of the soil. Usually normal soil has 8-10 ppm of cobalt whereas basic igneous rocks contain 20-100 ppm of cobalt. In serpentine ecosystem the average concentration of cobalt is more than 100 ppm (Peterson and Grilling, 1981).

2.4.3. Cobalt as trace element

Organisms need cobalt in nano-molar concentrations since they are part of vitamin B12 and act as coenzyme in biochemical reactions (Nies et al., 1992). It replaces manganese in activation of several enzymes and it replaces zinc in many biochemical reactions. In addition, it stabilizes the 3D structure of proteins, it is essential to maintain the osmotic balance in microbes thus maintaining the integrity of cell wall of bacteria (Ji and Silver., 1995). Besides, cobalt could be used to treat many ailments including anemia and certain infectious diseases. Cobalt plays a major role in maintaining and repair of the myelin sheath that surround nerve cells (Barceloux and Barceloux 1999). In addition, cobalt plays a significant role in the regulation and stimulation of certain enzymes, and is sometimes used by the body as a substitute for zinc (Kobayashi et al., 1999).

2.4.4. Industrial Applications of cobalt

Cobalt has been used for various applications such as production of alloys, electroplating, generation of gas turbines and petrochemical industries (Kuyucak and Volesky, 1989) Cobalt has many applications in various industries, for instance cobalt based alloys have been used for manufacturing combustion chambers in gas turbines (Coutsouradis et al., 1987). Additionally, it is used in dental and orthopedic implants
when combined with chromium (Kocijan et al., 2004). Mavrogenis et al., (2011), reported that (Co-Cr) metal alloy is effectively used for the maintenance of osteo-integration. Osteo-integration is direct structural and functional connection between bone and the artificial implant. Chemical and topographical surface of the (Co-Cr) metal implant plays a major role in the maintenance of osteo-integration in early stages of peripheral implant bone formation.

Ionizing radiation using $^{60}$Co is the used as an effective irradiation method (loahranu et al., 2004) to control the pathogenic microorganisms which are present in food (Monk et al., 1995). Performing such sterilization with minimum or negligible temperature rise is referred to as cold pasteurization.

**2.4.5. Cobalt in Nuclear Industries**

Nuclear power reactor’s spent decontamination solution contains dissolved corrosion deposits with significant amount of radioactivity (Rashmi et al., 2004). These dissolved corrosion deposits contain several gamma emitting radionuclides such as $^{51}$Cr, $^{59}$Fe, $^{58}$Co, $^{65}$Zn, $^{54}$Mn, and $^{60}$Co (Lin, 1996). In pressurized water reactor, large amount of low level radioactive wastewater is generated and these radioactive waste water has many radioactive isotopes such as $^{137}$Cs, $^{134}$Cs and $^{60}$Co (Szoke et al., 2005). Since this waste water is radioactive, it has to be removed to reduce the hazard of radiation exposure to the environment (as per IAEA safety standards). Among these various radionuclides removal, $^{137}$Cs removal can be done easily by ion exchange process, but the removal of $^{60}$Co is not possible since cobalt is more soluble when complexed with EDTA.
Among the various radionuclides, removal of $^{60}\text{Co}$ is very essential due to its longer half life (5.26 Years) and emission of higher gamma energies (1.17 and 1.33 MeV) (Jayarajan 

During decontamination process many organic acids and chelating agents such as citric acid, oxalic acid, EDTA and NTA were also added to the system which forms citrate, oxalate and EDTA complex of cobalt. Citrate and oxalate complex of cobalt can be easily removed by simple precipitation but not $[\text{Co(III)}\cdot\text{EDTA}]^-$. EDTA is a synthetic chelating agent which promotes the oxidation of Co(II) to Co(III) and forms $[\text{Co(III)}\cdot\text{EDTA}]^-$, as $[\text{Co(III)}\cdot\text{EDTA}]^-$ is easily soluble, it cannot be removed by simple chemical process (Brooks et al., 1999; Szoke et al., 2005)

2.5. Treatment methods of Heavy metals

Several techniques have been reported to treat the heavy metals, which are generated during industrial waste discharge. Treatment methods are categorized in to two broader divisions namely physicochemical and biological methods.

2.5.1. Physico-chemical treatment methods

Physicochemical treatment methods of heavy metals are including precipitation, adsorption, ion exchange, membrane and electrochemical technologies.

2.5.1.1. Precipitation

Metal precipitation can be achieved by the addition of coagulants, such as alum, lime, iron salts, and other organic polymers (Ahalya et al., 2003). This is the most effective and widely used process in industries (Ku and Jung, 2001). In this process, chemicals react with heavy metals to form insoluble precipitates and the formed
precipitates can be separated by sedimentation or filtration process using water. The main disadvantage of this process is generation of large amounts of sludge which contains many toxic compounds.

2.5.1.2. Adsorption

Adsorption defined as selective binding of metal soluble species that result in the immobilization of metals by adsorbent material. It is most widely used current method and it is highly suitable for waste water treatment due to the simplicity and cost effectiveness (Kwon et al., 2010). Adsorption occurs via various processes such as complexation, chelation, coordination by ion exchange, precipitation and reduction. Activated carbon is the most commonly used absorbent in this process (Wilson et al., 2006). Besides, clay mineral, zeolites, industrial solid wastes and fermentation products may also be used as adsorbents (Wang et al., 2008).

2.5.1.3. Ion exchange

Electrostatic force between the metal ions and exchange resin is the main factor responsible for ion exchange process in which, metal ions from dilute solutions are interchanged with ions held by weak forces on the exchange resin. High treatment capacity, high removal efficiency and faster kinetics make this method more efficient (Kang et al., 2004). Synthetic or natural solid resins could be used as ion exchange resins to exchange the cations with the metals in the waste water system. Among the various materials used as ion exchange resins, synthetic resins are commonly preferred as effective method, since they effectively remove heavy metals from the solution (Alyuz
and Veli, 20009). Besides synthetic resins, natural zeolites, naturally occurring silicate minerals are also have been used as ion exchange resin to remove the heavy metals from the waste water (Taffarel and Rubio, 2009).

2.5.1.4. Ultra filtration (UF)

In ultra-filtration, low trans-membrane pressure was used to remove dissolved and colloidal material in waste water. Pore size of UF membranes are larger than dissolved metal ions, hence these would easily pass through the membranes. To increase the metal removal efficiency, micellar enhanced ultrafiltration (MEUF) can be used. MEUF are usually used for removal of high organics and multivalent metal ions from water systems. Metal removal efficiency of MEUF depends on many factors such as concentration of metals, pH of the solution, ionic strength, and parameters related to membrane operation (Landaburu Aquirre et al., 2009). MEUF was used to remove many metals such as Cd\(^{2+}\), Cu\(^{2+}\), Ni\(^{2+}\), Pb\(^{2+}\) and Zn\(^{2+}\) from synthetic waste water (Sampera et al., 2009).

2.5.1.5. Electro-dialysis

Electro-dialysis involves the use of a semi-permeable membrane to separate the ionic components from waste water by application of electrical potential. This technique has been widely used for processing and treating of drinking water, brackish water and seawater. Apart from water treatment procedures, this technique can also be used in treatment of industrial effluents and recovery of useful materials from effluents (Sadrzaeha et al., 2009). For example, the removal of hexa-valent chromium from industrial effluent using set of ion exchange resins was successfully performed by Nataraj
et al., (2007). Efficiency of Cu and Fe removal using electro dialysis method was studied by Cifuentes et al., (2009). The major disadvantage of this method is the formation of the metal hydroxides, which clogs the membranes leading to reduced efficiencies of treatment.

2.5.1.6. Reverse osmosis

Reverse osmosis (RO) technique utilizes a semi-permeable membrane to separate the heavy metals by applying pressure greater than the osmotic pressure. It is one of the most commonly used methods used for the removal of wide range of dissolved species from water. Reverse osmosis process was used for the removal of Cu$^{2+}$ and Ni$^{2+}$ and the rejection efficiency of the two ions increased up to 99% using Na$_2$EDTA (Mohsen –Nia et al., 2007). A higher level of heavy metal efficiency was achieved using pilot scale membrane bioreactor combined with RO (Dialynas and Diamadopoulos, 2009). Higher cost, frequent restoration of membranes and power consumption due to the pumping will add up the disadvantages for this method.

2.6. Removal of cobalt by physicochemical methods

2.6.1. Physical adsorption of cobalt

2.6.1.1. Sepiolite

Sepiolite is a clay mineral complex of magnesium silicate and it has been used to remove cobalt using adsorption process. Efficiency of cobalt adsorption on sepiolite depends upon several factors such as activation condition, liquid to solid ratio, pH and temperature. Study of these multi-factors gives the information about the mechanism of
cobalt adsorption by sepiolite. Calculated values of free energy of adsorption, heat of adsorption and entropy of adsorption was used to conclude Co (II) adsorption on sepiolite is entrophically driven and it was characterized by physical adsorption (Kara et al, 2013).

2.6.1.2. Granular activated carbon

Efficiency of cobalt adsorption was checked using two different grades of granular activated carbon such as F 300 D and F 200 D in combination with P-nitro benzoic acid from aqueous solution. Adsorption isotherm of cobalt was calculated using Longmuir and Freundlich model to conclude adsorption of cobalt on the carbon was in monolayer. It was found that granular activated carbon F 300D was efficiently adsorbing cobalt than the F200 D (Hete et al., 2012).

2.6.1.3. Ion exchange resins

Ion exchange resins IRN77 and SKN1 were used to adsorb cobalt from the aqueous liquid. Initial pH, metal ion concentration and contact time were used to analyze the efficiency of these resins. It was found that optimum pH for the removal of cobalt was 5.3. Resins follow first order reversible kinetics for the removal of cobalt and the calculated equilibrium data for adsorption was fitted with both the Langmuir and Freundlich adsorption methods. In addition, adsorption of cobalt was affected by the presence of other metals (Rengaraj et al., 2002). Three cation exchange resins namely IRN77, SKN1 and IR 120 were characterized for the removal of cobalt ion from an aqueous solution by electro-deionisation. Ion exchange capacity, electrical conductivity and zeta potential of the resins were tested to evaluate the efficiency of cobalt removal.
From these experiments it was concluded that the resins removed more than 97% cobalt ions under optimal conditions (Yeon et al., 2003).

2.6.1.4. Polyurethane foam

Removal of cobalt ($^{60}$Co) from the synthetic intermediate level waste was also achieved using neat polyurethane foam (PU) as well as n-tributyl phosphate polyurethane foam (TBP-PU). It was found that PU foam comparatively extracted more cobalt than TBP-PU foam. In addition, extraction of cobalt was found maximum at 0.4M (Rao et al., 1997).

2.6.1.5. Co (II) imprinted polymer

Cobalt (II) imprinted polymer was synthesized and it was used to reduce the volume of waste generated during decontamination of nuclear reactors. The polymer was selectively removed the cobalt even in very less quantity i.e. parts per billion (ppb) levels. Cobalt adsorption and desorption using this polymer was rapid and this polymer was cobalt specific. Cobalt selectivity of the polymer was tested with radioactive cobalt containing 2 µCi wherein, it showed 55% reduction of cobalt activity (Bhaskarapillai et al., 2009).

Selectivity modification by metal ion imprinting technique was utilized to remove the cobalt in nuclear industry. Co (II) imprinted polymer was synthesized using epichlorohydrin as cross linker and used for selective removal of Co (II) in the presence of Fe (II). It was reported that imprinted chitosan selectively remove the Co(II) under various solutions conditions including typical nuclear reactor decontamination
formulation conditions containing strong complexants. Highest uptake of Cobalt (II) was achieved at pH 4.8 in citrate medium (Nishad et al., 2012).

2.7. Draw backs of physical methods

Negative side of physicochemical methods for the removal of pollutants has been discussed in many reports and can be summarized as follows.

* Heavy metals removal using chemical precipitation is ineffective when the metal ion concentrations are low. In addition, chemical precipitation is not economical and it produces large amount of sludge (Kongsricharoern and Polprasert, 1995).
* Ion exchange resins are expensive and it cannot be used to treat large volumes of waste water containing minimum quantity of heavy metal ions. In order to achieve high efficiency of heavy metal removal, ion exchange resins must be regenerated using various chemicals, subsequent discharge of these chemicals in outward generates secondary pollution.
* High cost of adsorbent material like activated charcoal makes the adsorption process very ineffective. High cost of membrane filtration technology, process complexity and membrane fouling and low permeate flux have limited the maximal use of membrane filtration technology for the removal of heavy metals from the waste water (Li et al., 2009).
* Physical methods such as coagulation, flocculation and flotation processes need high capital cost and maintenance. Besides it also requires various chemicals,
which in turn generates sludge, thereby making the above methods economically non viable.

In order to overcome these disadvantages, researchers have developed eco-friendly and cost effective remediation strategies to treat the heavy metal waste. In recent years there is tremendous scope for biological methods to treat many effluents that contains heavy metals.

2.8. Biological treatment methods

Treatment of heavy metals using biological system can be achieved by natural attenuation, bio-stimulation and bio-augmentation strategies.

2.8.1. Natural attenuation

Reduction in mass or concentration of compound in groundwater over time through biological process is called as natural attenuation. It is a natural process in which there is decrease in contaminants in soil and ground water. Effectiveness of this process is mainly depends upon physical, chemical, biological and hydro geological properties of the environment. This is the in situ process comprising biodegradation, dispersion, dilution, adsorption, chemical or biological stabilization, transformation and destruction of contaminants (USEPA, 1997).

2.8.2. Bio-stimulation

Bio-stimulation involves alteration of the environment to stimulate existing bacteria to enhance their bioremediation potential. Addition of various forms of nutrients, electron acceptor to stimulate the naturally occurring microbes present in the
environment (Scow et al., 2005). Addition of water, nutrient and oxygen in to the soil in order to increase the quality of microbes to degrade the pollutants is the main objective of the bio-stimulation. Depending upon the method of administration of nutrients, bio-stimulation is broadly categorized in to active and passive bio-stimulation. In active bio-stimulation electron donors, electron acceptors and trace elements are injected in to the environment to stimulate the natural organisms to increase the biomass resulting in degradation of the contaminant. In contrast, passive bio-stimulation simply spreads the nutrients on the surface of the contaminant site without any pumping.

2.8.3. Bio-augmentation

Bio-augmentation is the addition of specific microbes or consortia of microorganisms to the environment to increase the rate of degradation of the contaminant (Mrozik et al., 2010). Chemical structure, concentration and availability of pollutants as well as many physicochemical properties of the soil decides the efficiency of the process. Bio-augmentation is the most forceful method, since organisms are added to the contaminated environment rather than nutrients as in bio-stimulation process.

2.8.4. Bioaccumulation and Biosorption

Bioaccumulation is the metabolic dependent, energy driven process in which uptake of metals by living cells was observed. The toxicants could be transported into the cell, accumulate intracellularly across the cell membrane (Malik et al., 2004). Efficiency of this process is mainly depending upon the metabolic activities of the cell. Unlike bioaccumulation, biosorption is the passive uptake of toxicants by dead or inactive
biological materials or the materials derived from biological sources. Due to its simplicity, biosorption is being used as an alternate to many conventional methods (ion exchange, adsorption and precipitation).

2.9. Advantages of Biological methods

- **In situ** remediation – treatment of the contaminant material at the site was the primary advantage of the bioremediation process.
- Environmental friendly – Unlike chemical methods, biological methods do not generate any secondary pollutants, they are eco-friendly.
- Cost effective- Dead and low cost biological sources can be used as biosorbents

2.9.1. Comparison of Biosorption and Bioaccumulation

Difference of biosorption and bioaccumulation are listed below (Table- 2.3).

**Table 2.3: Comparison of biosorption and bioaccumulation**

<table>
<thead>
<tr>
<th>Characters</th>
<th>Biosorption</th>
<th>Bioaccumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cost</td>
<td>The biosorbents are derived from low cost natural products and industrial wastes and it is cost effective</td>
<td>Live cell related process makes the method costly</td>
</tr>
<tr>
<td>pH</td>
<td>Efficiency of this process is pH dependent</td>
<td>pH sensitive</td>
</tr>
<tr>
<td>Temperature</td>
<td>Temperature does not influence the process</td>
<td>The organism is live, hence temperature significantly affects the process</td>
</tr>
<tr>
<td>Selectivity</td>
<td>Poor response, however, it can be improved by modifying the surface of biomass</td>
<td>Moderate selectivity</td>
</tr>
<tr>
<td>Flexibility</td>
<td>More flexible</td>
<td>Not flexible</td>
</tr>
<tr>
<td>Rate of uptake</td>
<td>Rapid</td>
<td>Comparatively low, since, accumulation of metals will affect the efficiency rate of uptake is slow</td>
</tr>
<tr>
<td>Reusability</td>
<td>High, it can be used in many cycles</td>
<td>Low, since the toxicants are accumulated inside the cell</td>
</tr>
</tbody>
</table>

2.10. Bioaccumulation of cobalt
Interaction of cell surface components of microorganisms with metals, followed by the transport across the cytoplasmic membrane and intracellular localization are the prime factors for the bioaccumulation. Bio-adsorption followed by accumulation of cobalt is very useful since it makes the metal recovery process easy. Bioaccumulation of cobalt in estuarine microalgae *Chlorella salina* was demonstrated earlier by immobilizing the microalgae in calcium alginate beads (Garnham *et al.*, 1992). Adsorption of cobalt in microalgae was initially reported as a physical process and it was not affected by any of the physical factors such as light and temperature. Being a passive process, addition of metabolic inhibitors will not affect the efficiency of this process. Biosorption of cobalt occurs very rapidly and it was due to the ionic interaction between surface charge of cell wall moieties and the cobalt. Following adsorption there was uptake, which is an energy dependent process and mediated by the metal uptake system. Influence of various factors on cobalt adsorption in *C. salina* was extensively studied and it was reported that cobalt adsorption increased with increasing cell density whereas it has decreased the specific amount of cobalt taken up by the cells. Following adsorption desorption is a necessary process, since metal adsorbed to biomass will block further adsorption due to the lack of free binding sites and this can be minimized by desorption. Effect of sodium chloride on desorption of cobalt was studied and the results have shown that 0.5 M NaCl and low pH decreases desorption of the cobalt (Garnham *et al.*, 1992). Bioaccumulation of radionuclide such as cobalt ($^{57}$Co) and Cesium ($^{137}$Cs) was studied using five different marine phytoplankton species with different growth conditions. Among the growing cells,
*E. huxleyi* was able to uptake high amounts of cobalt until it reached 1mBq/cell. For non-growing cells the uptake was highest in *Thalassiosira pseudonama* (Heldal *et al*., 2001).

Contamination kinetics is used as a vital factor to understand the significance of radionuclide contamination and their impact on the environment. It was studied using two different fresh water algal components such as *Scenedesmus obliquus* and *Cyclotella meneghiana* derived from radioactive contaminated natural suspended soil. It was found that *C. meneghiana* was more efficient in adsorbing cobalt than *S. obliquus* (Adam *et al*., 2003).

2.11. Techniques used to study cobalt adsorption

A detailed mechanism of cobalt adsorption was studied using various physicochemical techniques in *Rhodobacter sphaeroides* R26. Metabolic inhibitors were used to study the active and passive mode of cobalt adsorption, since passive adsorption was energy independent process and it was not blocked by metabolic inhibitors. Role of cell envelope in cobalt uptake and role of specific charged functional groups on adsorption was studied using acid – base titrations, fourier transform infrared spectroscopy (FTIR), and attenuated total reflection fourier transform infrared spectroscopy (ATR-FTIR). Mechanism of cobalt uptake, and accumulation was studied using inductively coupled plasma emission spectroscopy (ICP-AES) (Italiano *et al*., 2009).

2.12. Bacteria isolated from onsite polluted areas are used as ideal bioremediation agents
Organisms isolated from contaminated sites or industrially polluted sites have been used as ideal bioremediation agents as they have developed tolerance to survive in such harsh environments. Microorganisms undergo constant and continuous exposure to adverse conditions like heavy metals or any other pollutants and undergo physiological and genetic adaptation over a period of time and the organism can develop the potential to overcome such adverse conditions (Gadd, 1993). Reports on four pure bacterial cultures *Kocuria palustris*, *Micrococcus luteus*, *Ochrobacterium species* and *Pseudomonas aeruginosa* isolated from spent nuclear pool water of interim spent fuel storage in Slovak Republic showed bioaccumulation potentials for $^{60}$Co and $^{137}$Cs. After 48 hours cultivation in mineral medium, maximum uptake of $\text{Cs}^+$ was upto 7.54 µmol/gm by *Ochrobacterium Spp.*, 19.6µmol/gm by *M. luteus*, 20.1 µmol/gm by *K. palustris* and for $\text{Co}^{2+}$ was 31.1µmol/gm (*Ochrobacterium spp.*), 86.6 µmol/gm (*M. luteus*), 16.9 µmol/gm (*K. palustris*) respectively (Tisakova *et al.*, 2013).

2.13. Bio-magnification of cobalt

Nutrient recycling is the essential factor to maintain the self-sustainability of the ecosystem. Continuous transfer of nutrients from low trophic levels such as decomposers to high trophic levels by consumers is essential to maintain nutrient recycling. During nutrient recycling some contaminants also get transferred and it affects the homeostatic conditions of ecosystem. Studying the bioaccumulation of pollutants over the different trophic level was essential to know the health of the ecosystem. Dynamics of cobalt in various trophic levels is essential to know the passageway of cobalt transfer and their
control. Synthetic waste water contaminated with cobalt was used to analyze cobalt bioaccumulation in silk worm (Bombyx mori) food chain (Ashfaq et al., 2009). Soil irrigated with waste water was used to cultivate mulberry leaves and silkworm was allowed to consume the leaves. Samples were collected from waste water, soil, mulberry and silkworm to analyze the cobalt concentration by atomic absorption spectrophotometer. Silkworm was used as a pollution indicator of cobalt as its body weight, body length and body mass was found to be related to the cobalt concentration. The maximum amount of cobalt found in waste water, soil, mulberry, and silkworm at pH 4.5 was 400, 273.5, 42.85, and 36.62 mg/kg respectively (Ashfaq et al., 2009).

2.14. Phyto-remediation of cobalt

Phyto-remediation is a process in which plants are used to sequester or remove or degrade pollutants which are present in soil, sediments, surface water and ground water. The prime factors which determine the efficiency of phyto-remediation are pH, initial concentration of pollutant, intensity of light, amount of biomass and radioactivity. Removal of effluent contaminated with cesium ($^{137}$Cs) and cobalt ($^{60}$Co) was studied using Eichhornia crassipes and it was found that uptake of active cesium from the stimulated spent solution was inversely proportional to initial radioactivity and directly proportional to the increase in mass of plant and the duration of light exposure. Moreover, uptake efficiency of cesium in mixed solution was comparatively higher than those individual species, but the uptake of cobalt was affected by the presence of cesium (Saleh, 2012). Effect of pollutants on various parts of the plant was important to design
phyto-remediation experiments, since various parts of the plant accumulated varying amount of pollutants and it was mainly depending upon the physiological status and the chemical nature of the pollutant. Bioaccumulation of radioactive cobalt \(^{(60)}\text{Co}\) by tobacco was studied under hydroponic growth conditions. Roots and shoots of the plants were the major cobalt accumulation sites and it accumulates 580 and 50 \(\mu\)g cobalt/gm of biomass respectively. On the contrary presence of 0.5 mM of EDTA and NTA increased cobalt uptake by 86% in shoots rather than roots (Hornik et al., 2006). In addition, young leaves of the tobacco accumulate less amount of cobalt than matured leaf.

2.15. Biosorption of cobalt

Biosorption is mainly a physical, passive and a metabolism independent process, where metal binds to live or dead biomass. The process is comparatively rapid and can be reversible. Physicochemical interaction between the metals and the charged functional group moieties which are present on the surface of the cell wall and extra cellular polymeric substances (EPS) are the key factors responsible for the biosorption.

2.15.1. Biofilm mediated Biosorption

Biofilm can be defined as a microcosm of bacterial consortium having biotic constituents in an exopolymeric matrix at interfacial regions (between the organism and substrate) (Costerton et al., 1978). They are very common in environments like water, soil, living tissue and industrial water distribution systems. Biofilms mostly constitutes the extracellular polysaccharide (EPS) (Chandki et al., 2011). EPS are mainly rich in water, carbohydrate, protein and uronic acids (Jain et al., 2013). EPS being a charged
moiety effectively adsorbs heavy metal ions and are extensively used for bioremediation purpose. To study the adsorption of cobalt immobilized EPS of activated sludge bacterium *Chryseomonas luteola* TEM05 was used in various combinations of biosorbents such as alginate, alginate + EPS, alginate + biomass, alginate+ EPS + biomass. It was shown that maximally 150 mg of cobalt / gm of biomass adsorbed by alginate +EPS. The order of cobalt removal of various biosorbents were found to be alginate –EPS,> alginate-biomass>, alginate –EPS-biomass,> alginate (Ozdemir *et al*., 2005). Effect of increasing amounts of EPS on the adsorption efficiency of cobalt was significant in achieving optimum cobalt biosorption. EPS of marine bacterium *Enterobacter cloaceae* adsorbed 8% of cobalt through biosorption. It was reported that increasing amounts of EPS from (0.27mg/g - 4.38 mg/g of EPS) increased cobalt adsorption from 25- 100 mg /l (Iyer *et al*., 2005).

2.15. 2. Biomass mediated Biosorption

Biosorption is a simple physical phenomenon and its efficiency varies depending upon many factors such as pH, amount of biosorbent, initial concentration of solutes, temperature and availability of other metals. Effect of various factors on adsorption of cobalt was effectively studied in cyanobacteria *Oscillatoria auguistissima*. This cyanobacterium adsorb significant amount of cobalt within 15 minutes of contact time and it followed Freundlich model for the adsorption. (Ahuja *et al*., 1999). Moreover, adsorption of cobalt by this organism was pH dependent and temperature independent and was affected by the presence of Mg$^{2+}$, Ca$^{2+}$, nitrate and sulphate. The mechanism of cobalt
adsorption was ion-exchange type and maximum desorption (76%) was reported in the presence of Mg$^{2+}$ and 1.0 mM Na$_2$CO$_3$ (Ahuja et al., 1999).

Strong adsorption behavior of microbes towards the metal ion is the function of many factors such as temperature, pH, chemical makeup of the organism and etc. Equilibrium and kinetic rate of cobalt adsorption gave the information about the efficiencies of biosorbents and their wide application in industries. Equilibrium and rate of cobalt absorption was studied using fungal biosorbent PFB 1, this biosorbent uptakes 190 mg g$^{-1}$ of cobalt. This was five times higher than earlier reports of fungal based biosorbents. Increasing temperature showed negative impact on cobalt adsorption since equilibrium uptake was decreased with increasing temperature in the range of 30-45°C. The highest uptake of cobalt was reported at pH 7. Mass transfer control models were used to study the rate of cobalt uptake and resistant to mass transfer of metal ions. External mass transfer coefficient of cobalt adsorption on PFB was found to be 0.075 m s$^{-1}$. As cobalt biosorption occurred due to ion exchange method, EDAX and EPR studies were conducted to elucidate the mechanisms. Recurrent use of biosorbent was tested by desorption and readsorption studies with acidic, basic and salt desorbents. Highest desorption 92.5% and 70.5 % were reported at the end of first and third cycles respectively using 0.1N hydrochloric acid as desorbent (Suhasini et al., 1999).

2.16. Adsorption equilibrium models

Adsorption is a process in which solute accumulates on the surface of the solid and it is expressed in the form of isotherm. Adsorption isotherm gives the information
about the nature of molecule distribution between the liquid and solid phase when the adsorption process reaches the equilibrium state (Nwabanne and Igbowke, 2008).

Various models have been used to describe the adsorption reactions. They are generally described in two broader categories

- Empirical model
- Mechanistic model

2.16.1. Empirical Model

The empirical model provides the information about adsorption without theoretical background. These models are framed based upon simple mathematical relationships characterized by number of parameters, which gives the information about the experimental behavior over the wide range of operating conditions (Esposito et al., 2002).

For single solution adsorption equilibrium, two models that were most widely used were as follows

1. Langmuir model
2. Freundlich model

2.16.1.1. Langmuir model

The Langmuir model is a theoretical model for monolayer adsorption where the distribution of metals between the solid and solute interface has been derived using Langmuir equations (Namasivayam et al., 1998).

\[
\text{Langmuir: } q = \frac{q_{\text{max}} b \text{Ceq}}{1 + b \text{Ceq}}
\]
Whereas,

q – Milligram of metal accumulated per gram of the biosorbent material

Ceq – Metal residual concentration in solution

$q_{\text{max}}$ – Maximum specific uptake corresponding to the site saturation

b - Ratio of adsorption and desorption rates

**2.16.1.2. Freundlich model**

Another empirical model for monolayer adsorption is

\[ q = K_F \cdot \text{Ceq}^{1/n} \]

Where, $K_F$ and $n$ are constants which are indicators of adsorption capacity and intensity respectively (Aksu et al., 2003).

These models can be applied at a stable pH and it can be used for modeling the monolayer adsorption. The values of the Freundlich and Langmuir parameters were obtained from the linear correlation between the values of $\text{Ceq}/q$ and $\text{Ceq}$ and $\log q$ and $\log \text{Ceq}$ respectively. Efficiency of the above said models mainly depend upon two factors such as nature of the sorbent material and their multiple active sites and complex solution chemistry of metals.

Besides equilibrium batch sorption test for assessment of sorption performance, process oriented studies and their kinetics could be tested using dynamic continuous flow tests.

**2.16.2. Mechanistic model**

Mechanistic model is used to describe the solute adsorption on to the surface of biomass (Hass et al., 2001). Biomass characterisation along with the formulation of a set
of hypothesised reactions between adsorbent sites and solutes were used for the development of this model. Apart from biomass characterisation, solution chemistry of the solutes also considered significantly for the development of this model. This model was used to analyse the biosorption in several investigations with considerable success. (Yun et al., 2001)

2.17. Dissimilatory metal reduction

Microbes are ubiquitous in nature; they perform metal reduction in various environmental conditions from aquatic environment to terrestrial surface. Diverse group of microorganisms were involved in reduction of ferric, uranium, selenium, chromium, technetium and other metals. By reducing the metal microbes not only conserve the energy but also act as ideal bioremediation agents (Lovley et al., 1991). This is a microbial mediated process to conserve the energy by oxidizing (organic or inorganic substances) through an electron donor to reduce a metal (electron acceptor). They use the metal as final electron acceptor and have a significant impact on maintaining bio-geo chemical cycle of the Earth. In nuclear industry, a wide variety of multivalent metals exhibit a series of threats to the environment. The oxidized form of the metals such as Fe$^{3+}$, U(VI), Cr, (VI), Tc(VII) and Cobalt complex [Co(III)-EDTA]$^-$ are highly soluble in aqueous systems and ground water. Once they get reduced their solubility is decreased and they can be precipitated.

2.18. Reduction of heavy metals by bacteria
2.18.1. Iron reduction

Reduction of iron is most important since it is responsible for respiration in primitive form of life and could be basis for life on other planets (Nealson et al., 2002). Microbes carrying out Fe reduction can also reduce many heavy metals and toxicants, thereby reducing their toxicity (Vargas et al., 1998). In subsurface environments, Fe(III) is the dominant electron acceptor for microbial respiration. Diverse group of microorganisms can reduce the Fe metal in the presence of sugars or amino acids as electron donor, likewise in Vibrio sp. (Jones et al., 1984), Wolinella succinogenes (Lovley et al., 1988) and Archaea such as Archaeoglobus fulgidus, Pyrococcus furiosus and Pyrodictium abyssi (Vargas et al., 1998). These organisms can couple the oxidation of hydrogen and short-chain fatty acids to the reduction process. Besides these organisms sulfate-reducing bacteria can also reduce Fe (III) (Coleman et al., 1993) and other metals. Shewanella oneidensis (previously Alteromonas putrefaciens and then Shewanella putrefaciens) and Geobacter metallireducens (formerly strain GS-15) was the first known organism to conserve the energy for growth through reduction of Fe (III) (Lovley et al., 1988; Myers et al., 1993). Acetate oxidizing Fe (III) reducer Shewanella putrefaciens conserve the energy to support the growth by oxidizing organic compounds with reducing Fe (III). Numerous microbes (Pseudomonas sp, S. putrefaciens, Shewnella alga BrY) can couple the oxidation of H$_2$ to the reduction of Fe (III) (Fischer et al., 1988; et al., 1988; Lovley, 1991)
2.18.1.1. Mechanism of Fe (III) Reduction

The involvement of c-type cytochromes in Fe (III) reduction were reported in electron transport from Fe (III) to metals. (Lovley et.al 1993; Naik et al., 1993; Gaspard et al., 1998; Lloyd et al., 2003; Magnuson et al 2000; Myers et al 1993 & 1997). Besides direct transfer of electron from Fe (III) to metals, soluble electron shuttles also play a major role in electron transfer between metal reducing bacteria and mineral surface. For example, humic substances and other extracellular quinones were used as electron acceptor by Fe (III) reducing bacteria (Lovley et al., 1996). Subsequently, reduced quinones transfer electrons to Fe (III) and oxidized humics carry out the next cycle of reduction of Fe(III) (Nevin et al., 2002). Minimum quantity of electron shuttle, for example 100 nM of humic analogue anthraquinone desulfonate (ADS) is enough to accelerate reduction of Fe (III) (Seeliger et al., 1998). Through several experiments, it was reported that involvement of c-type cytochromes as electron carriers in Fe (III) reduction in D. acetoxidans (Roden et al., 1993). Enzymes of Thiobacillus ferrooxidans can reduce the Fe (III) under acidic conditions when reduced sulfur components acts as electron donor. Electro-phoretically purified periplasmic space enzyme and plasma membrane oxidized sulfide to sulfite with Fe (III) act as electron acceptor (Sugio et al., 1989).

2.18.2. Reduction of Uranium

Anaerobic microbial reduction of soluble U (VI) to insoluble U (IV) is the promising strategy, which can be used to prevent the immobilization of uranium in aquatic water bodies. First report of uranium reduction was by Lovely and coworkers
using Fe (III) reducing bacteria *Geobacter metallireduceens* (Lovley *et al.*, 1991). Besides Fe(III) reducing bacteria and some of the non Fe (III) reducing bacteria such as *Clostridium sp* (Francis *et al.*, 1994), sulfate reducing bacteria *D. desulfuricans* (Lovely *et al.*, 1992) also actively involved in the reduction of uranium but they are not able to conserve energy for growth via reduction process. Uranium bioremediation was carried out by stimulating the anaerobic microbes which were involved in removal of uranium from the contaminated sites. Several reports had been seen regarding the anaerobic reduction of uranium by environmental microorganisms. Among them involvement of cell free extracts of the *Veliionella atypica* was the first evidence reported for the reduction of uranium along with variety of other metals (Woolfolk *et al.*, 1962). Apart from the above said *G. metallireduce*, *S. putrefaciens*, and several *Desulfovibrio* species were also involved in reduction of U (VI) to (IV) (lovley *et al.*, 1992).

### 2.18.2.1. Involvement of spores in uranium reduction

In order to find out the involvement of spores in metal reduction of uranium, various types of cells such as pasteurized spores, unpasteurized spores, glutaraldehyde fixed spores, pasteurized vegetative cells and live vegetative cells were used and their uranium reduction potential was measured in spent fermentative medium, Fresh WLP medium and spent medium purged with and without H2 and N2 was used in these experiments, it was reported that spores are directly involved in the reduction of uranium when H2 was used as an electron donor. It was also reported that unknown factors that are less than 3 kDa produced by the cells are required for the reduction of uranium (Junier *et
al., 2009; Vecchia et al., 2010).

2.19. Reduction of selenium

In nature, Selenium exists as Se (VI) selenate ($\text{SeO}_4^{2-}$), Se (IV) (Selinite, $\text{SeO}_3^{2-}$), Se (0) elemental selenium and Se (-II) (selenide). Heterotrophic organisms such as *Clostridium*, *Citrobacter*, *Flavobacterium* and *Pseudomonas species* reduce the selenate to elemental selenium (Bautista et al., 1972; Kauffman et al., 1986; Burton et al., 1987).

2.20. Reduction of $[\text{Co(III)-EDTA}]^-$

Radioactive cobalt $^{60}\text{(Co)}$ is a significant constituent of radioactive waste and it is easily transported to groundwater when it is combined with ligand such as EDTA. In chelated form, cobalt is more mobile than non-chelated from. Mobility of cobalt could be due to intentional or accidental co-release when it is combined with synthetic organic chelating agent such as EDTA (Riley et al., 1992; Killey et al., 1984; Means et al., 1978). Since oxidized form of cobalt is more mobile and stable it has to be removed prior to the disposal. Transformation of oxidized, soluble, highly toxic and mobile form of Co[(III) EDTA]$^-$ in to reduced, insoluble, less toxic and non transportable form by reduction is the most promising strategy that is used for $[\text{Co(III)-EDTA}]^-$ remediation in nuclear waste decontaminated sites. Properties of $[\text{Co(III)-EDTA}]^-$ and $[\text{Co (II) EDTA}]^{2-}$ are listed in Table 2.4.

**Table 2.4: Properties of $[\text{Co(III)-EDTA}]^-$ and $[\text{Co(II)EDTA}]^{2-}$**

<table>
<thead>
<tr>
<th></th>
<th>$[\text{Co(III)-EDTA}]^-$</th>
<th>$[\text{Co(II)-EDTA}]^{2-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twenty five times more thermodynamically stable (Ogino et al., 1983)</td>
<td></td>
<td>Less stable</td>
</tr>
<tr>
<td>Kinetically inert to exchange with other</td>
<td></td>
<td>Easily exchangeable with other metals in</td>
</tr>
</tbody>
</table>
metals in solution (Margerum et al, 1978) | solution  
---|---
Highly mobile in saturated subsurface environment (Olsen et al., 1986) | Immobile  
Not dissociated easily | Dissociates in the presence of cationic metals such as Al or Fe at pH below 6.5 and displaced cobalt is immobilized in the presence of oxide metals, (Girvin et al., 1993; Szecsody, 1994)  
Show more affinity towards EDTA | Show less affinity towards EDTA

2.20.1. Reduction of $[\text{Co(III)}\text{-EDTA}]^-$ by *Shewanella alga* BrY

Reduction of $[\text{Co(III)}\text{-EDTA}]^-$ by dissimilatory metal reducing anaerobic bacteria *Shewanella alga* BrY (BrY) was demonstrated using column flow experiments. This bacteria coupled growth with the reduction of $[\text{Co(III)}\text{-EDTA}]^-$ when lactate used as electron donor. Reduction of $[\text{Co(III)}\text{-EDTA}]^-$ in column flow experiments form less stable, less mobile $[\text{Co(II)}\text{-EDTA}]^{2-}$. Microbial reduction in the presence and absence of manganese oxide was also studied with these bacteria (Gorby et al., 1998). Brooks et al., (1999) conducted bacterial reduction of $[\text{Co(III)}\text{-EDTA}]^-$ in the presence of geochemical oxidant using *Shewanella alga* growing in lactate medium. They conducted column experiments to understand the mechanism of reduction of $[\text{Co(III)}\text{-EDTA}]^-$. This bacterium performs the reduction evidenced by delay in $[\text{Co(III)}\text{-EDTA}]^-$ transport in column in presence of suitable minerals adsorbent such as (Fe(OH)$_3$) since formed $[\text{Co(II)}\text{EDTA}]^{2-}$ get adsorbed on Fe hydroxide subsequently Fe(III) displaces cobalt and delay the transport of cobalt. In addition this bacterium performed the $[\text{Co(III)}\text{-EDTA}]^-$ reduction even in the presence of strong oxidizing agent such as MnO$_2$ (Brooks et al., 1999).
2.20.2. Reduction of [Co(III)-EDTA]⁻ by Shewanella at Extreme conditions

Metal reduction at cold temperatures by Shewanella was also been reported (Stapleton et al., 2005). This bacteria utilize [Co(III)-EDTA]⁻, Manganese oxide and various forms of iron as electron acceptors for their growth in the presence of lactate under the anaerobic conditions at 8 ºC (Stapleton et al., 2005). Reports suggest that bacterial metal reduction not only occurs in room temperature, but also occurs in extreme cold and thermal temperatures. Thermophillic bacterium Shewanella was isolated from deep subsurface sediment and -its metal reduction ability was tested using various metals such as Fe(III), Co(III) and Cr(IV). It was found that in extreme temperature this bacterium enhances metal reduction up to four folds by nonspecific mechanisms.

2.20.3. Mechanism of [Co(III)-EDTA]⁻ reduction by Shewanella oneidensis MR-1

Mechanism of [Co(III)-EDTA]⁻ reduction by Shewanella oneidensis MR-1 was proposed by Hau et al., (2008). This bacterium reduced [Co(III)-EDTA]⁻ in the presence of 20 mM lactate as electron donor and 5 mM [Co(III)-EDTA]⁻ as electron acceptor. Growth of the bacterium was not coupled with reduction of [Co(III)-EDTA]⁻ due to toxicity of [Co(II)-EDTA]²⁻ and this was evidenced by restoration of growth when 25 mM MgSO₄ was added. Reduction of [Co(III)-EDTA]⁻ to [Co(II)-EDTA]²⁻ by this bacterium need Mtr extracellular pathway and associated pathways which are needed to develop functional enzymes. Mtr enzymes are c-type cytochrome maturation enzymes involved in proper localization and secretion of enzymes (Type-II secretion) involved in [Co(III)-EDTA]⁻ reduction (Hau et al., 2008).
2.20.4. Metal reduction model

Metal reduction model gives the information about coupled transport and oxidation-reduction of pollutants. A model was developed based on published data on the movement of Co-EDTA on the columns packed with manganese oxide coated sand. This model was used to solve equations for the movement of [Co(II)-EDTA]\(^2^-\), [Co(III)-EDTA] and dissolved oxygen due to the bulk motion. In addition these equations were used to describe oxidation of [Co(II)-EDTA]\(^2^-\) to [Co(III)-EDTA] by manganese oxide (Saiers et al., 2000). Another metal reduction model to study the metal reduction kinetics of *Shewanella oneidensis* MR-1 was developed based on Michaelis–Menten equation. Time series data from the various aqueous species of metals including [Co(III)-EDTA] were used in this model. This model uses real-time estimated parameters to predict the reduction rate of metals at varying concentrations (Lall et al., 2007).

2.20.5. Reduction of [Co(III)-EDTA] in the presence of MnO\(_2\)

Reduction of [Co(III)EDTA] was observed in *Shewanella alga* BrY (BrY) even in the presence of strong oxidant like manganese dioxide, where the bacteria reduced cobalt by using metal reducing enzymes. In natural systems, bacterial reduction of [Co(III)EDTA] with manganese (IV) oxide was significant, since it is coupled with the oxidation of [Co(II) EDTA]\(^2^-\) as a result of biotic-abiotic cycle between [Co(II)-EDTA]\(^2^-\) and [Co(III)-EDTA]. Reduction of [Co(III)-EDTA] by microorganisms and the geochemical oxidation of [Co(II)-EDTA]\(^2^-\) determines the fate and transport of [Co(II)-EDTA]\(^2^-\) in the environment, (Brooks et al., 1999).
2.20.6. Reduction of [Co(III)-EDTA]⁻ by *Desulfovibrio vulgaris*

Direct (enzymatic) and indirect (metabolic mediated) reduction of [Co(III)-EDTA]⁻ using *Desulfovibrio vulgaris* was reported (Blessins and Toad, 2001). Here, [Co(III)-EDTA]⁻ reduction was not coupled with growth of bacteria, however [Co(III)-EDTA]⁻ acted as a competing electron acceptor and limits the cobalt reduction. When sulfate was introduced with this bacterium, it enhanced [Co(III) EDTA]⁻ reduction by forming sulfide, while sulfide formation reduces the [Co(III)-EDTA]⁻ into [Co(II)-EDTA]²⁻ in a pathway involving polysulfide formation and leads the cobalt sulfide precipitation (Blessing and Toad, 2001).

2.21. Bioremediation using aerobic granules

2.21.1. Significance of granules in bioremediation

Biogranules are compactly packed dense granules containing microbial consortia, which, consists of heterogeneous group of bacteria. Biogranulation is the integrated phenomenon driven by biological, physical and chemical interactions (Liu et al., 2004). Each gram of granule is crowded with millions of microbes, which are performing complex roles in degrading industrial wastes. Biogranules have a highly dense, strong compact structure with a regular spherical shape have fast settling abilities, which are comparatively more efficient than the conventional activated sludge process (Liu et al., 2004).

2.21.2. Types of Biogranules

Based upon the mode of respiration biogranules are categorized in to two
divisions such as anaerobic granules and aerobic granules

2.21.2.1. Anaerobic granules

Up flow anaerobic sludge blanket (USAB) was used to study the formation of anaerobic granules (Alves et al., 2000). An anaerobic granule was used to treat high strength waste water contaminated with soluble organic pollutants (Lettinga et al., 1980). Numerous models were developed over the period of two decades to understand the mechanism of anaerobic granulation such as inert nuclei model, divalent cation bridge model, proton translocation- dehydration model, extracellular polymer model, spaghetti model, syntrophic micro-colony model, thermodynamic models and quorum sensing models (Liu et al., 2003i). Despite anaerobic granules having high potential to treat waste water it also has some drawbacks. Major drawback of anaerobic granules were; need for a long start up time (2-4 months or even longer time) besides, it need high operation temperatures and unsuitable for low strength waste water. Moreover, this is not suitable for the removal of nitrogen and phosphorous from the waste water (Liu et al., 2004).

2.21.2.2. Aerobic granules

To overcome the above said disadvantages in anaerobic granulation technology, studies has been carried out to develop aerobic granulation technology. First report of development of aerobic granules came from Mishima and Naamura (1991) using continuous aerobic up flow sludge blank reactor. Aerobic granules consisted of a huge consortium of microbes containing millions of individual bacteria. As a group they are efficient enough to degrade diverse group of pollutants, but none of the pollutants can be
degraded by individual species. Aerobic granules were developed using sequencing batch reactor (SBR) (Etterer and Wilderer., 2001) which are modifications of the conventional activated sludge reactor used for waste water treatment (Jiang et al., 2002; Moy et al., 2002; Lin et al., 2003). Aerobic granulation is a step by step process involving the sequence of events starting from sludge to compact aggregates, subsequently granular sludge and finally to mature granules (Morgenroth et al.,1997; Beun et al.,1999; Peng et al.,1999; Tay et al., 2001a).

2.21.3. Development of aerobic granules

Bio-granulation was the collaborative phenomenon performed by biological, physical and chemical interactions (Liu et al., 2004). Biofilm is the essential factor which need for the growth of aerobic granules (Liu and Tay 2002; Yang et al., 2004a). It was multistep process and a number of conditions are involved in the formation of aerobic granules. Initial force which is needed for the granular development was the cell to cell contact (Tay et al., 2002c), and it was initiated by many factors such as hydrodynamics, diffusion mass transfer, gravity, thermodynamic effects and cell mobility. This is followed by physical contact, stabilization of the cell–cell contact, which occurs due to the interaction of physical, chemical and biochemical forces. Van der Wall’s forces, ionic interaction, forces involving in the reduction of surface free energy, surface tension and hydrophobicity were the significant physical forces involved in stabilization of multicellular contacts. Apart from this physical forces few chemical and biochemical forces such as dehydration of cell surface, fusion of cell membrane, quorum sensing mediated
signaling were also involved in maintenance of cell to cell contact. Upon establishing the cell to cell contact there was maturation of cell aggregation, triggered by multiple factors such as EPS, growth of cellular clusters, metabolic products and environmentally driven genetic factors resulting in the development of highly organized microbial structure. This highly organized steady state three dimensional structure of microbial aggregate is also due to hydrodynamic shear forces (Tay et al., 2003; Chisti, 1999a; Tay et al., 2001c; Qin et al., 2004).

2.21.4. Role of hydrophobicity in the formation of aerobic granules

Hydrophobicity of cell surface is the main factor which initiates the aerobic granule formation and was explained using thermodynamic principles. Hydrophobicity of cell surface was inversely proportional to the Gibbs free energy of the surface which promotes the cell-cell interaction followed by self-aggregation of bacteria. The initial process in granular development was initiated by hydrophobicity of the cell surface leading to stronger cell-cell interaction thus forming dense structure. Subsequently, EPS plays a very important role in the cell adhesion and cohesion, which is vital in maintaining the structural integrity of the microbial consortia (Pringle and Fletcher, 1983; Bos et al., 1999; Liu et al., 2003c;2003h).

2.21.5. Characteristics of aerobic granules

Granules differ from the conventional activated sludge by the following properties (Liu et al., 2004).

* Denser and stronger microbial structure
* Regular, smooth, round and clear outer surface
* Able to seen as clear entity in mixed liquor during mixing and settling phases
* High biomass retention and faster settling property
* Able to withstand high organic loading rate
* Less vulnerable to the toxicity of organic chemicals and heavy metals in waste water

2.21.5.1. Morphology of granules

The shape of aerobic granules is spherical with clear outer surface. Size of the granule is an important factor required to characterize the health of aerobic granules. Average diameter of granules varies in the range of 0.2 to 5 mm. Equilibrium between growth and detachment of granule due to the hydrodynamic shear force determines the diameter of granules (Peng et al., 1999; Tay et al., 2001a, c; Zhu and Wilderer, 2003).

2.21.5.2. Settleability

Settling ability in granules is an ideal parameter to assess the health of aerobic granules (Qin et al., 2004). Short setting time of granules is essential for fast settling of bacteria and their optimum growth. Granules with poor settling property can be easily washed out from the system. Efficient solid-liquid separation depends upon the faster settling properties of the granules hence granules with excellent settling property are required for effective removal of pollutants from waste water. Usually settling time of healthy aerobic granules occurs within a minute (Liu et al., 2003f; Qin et al., 2004; Beun et al., 2000; Tay et al. 2001b).
2.21.5.3. Density and strength

Integrity coefficient is an index to measure the physical strength of granules, measured as ratio of residual granule to the total weight of the granular sludge. Physical strength of granules is essential since, it gives protection against high abrasion and shear force. Generally specific gravity of matured aerobic granules ranges from 1.004 to 1.065 (Etterer and Wilderer, 2001; Tay et al., 2001b).

2.21.5.4. Biomass retention

Operation of sequencing batch reactor (SBR) has continuous cycle of feeding, aeration, settling and effluent discharge. This cycle time gives the information about frequency of solid discharge through effluent withdrawal (washout frequency) and it is depend upon the hydraulic retention time (HRT). HRT defined as volume of effluent discharged divided by the working volume of the SBR. Frequent running of cycle is advantageous for one side since, it would suppress the growth of suspended solids due to the frequent wash out, however, on other side it would be disadvantageous because of frequent wash out inhibits the growth of bacteria. Since ideal granules have the balanced HRT, which should be short enough to prevent the settling of suspended solids and long enough to support the settlement and growth of bacteria (Tay et al., 2001a; 2001b).

2.22. Major factors affecting the development of microbial granulation

Formation of aerobic granules controlled by many factors, they are as follows:

2.22.1. Type of carbon source

Glucose, acetate, ethanol, phenol and synthetic waste water were used as carbon
source to cultivate the aerobic granules. Microstructure of granules and diversity of immobilized bacteria varied with the type of carbon source used for their cultivation. It was found that glucose grown granules exhibited filamentous structure, while acetate grown granules shown non filamentous, compact bacterial structure with rod like species (Beun et al., 1999; Peng et al., 1999; Tay et al., 2001a; Moy et al., 2002; Jiang et al., 2002).

2.22.2. Organic loading rate

Role of organic loading rate in the formation of granules was tested and the results were reported by many researchers. It was revealed that aerobic granules had organic loading rate ranging from 2.5 to 15 kg COD/m$^3$/day and formation of granules were not affected by organic loading rate. Physical characteristics of the granules depend upon the organic loading rate and it was evidenced by increased size of granules from 1.6 to 1.9 mm with the increase of organic load from 3-9 kg COD/m$^3$/ day. Physical strength of the granules decreased with increase of organic loading rate (Moy et al., 2002; Liu et al., 2003a).

2.22.3. Hydrodynamic shear force

Hydrodynamic shear force is an essential factor which determines the aerobic granulation. Granule density and strength is directly proportional to the hydrodynamic shear force. At high hydrodynamic shear force more regular, rounder and compact granules were developed. Formation of aerobic granules triggered only above superficial air velocity above 1.2 cm/s in a column SBR (Shin et al., 1992; Tay et al., 2001a).

2.23. Applications of aerobic granules
2.23.1. Waste water treatment

Application of aerobic granules in high strength waste water treatment is well reported. Gradual increasing of organic loading increases the COD removal efficiencies up to 89%. Glucose fed aerobic granules gradually exposed with organic loading rate raised from 6-9, 9-12 and 12-15 kg COD/m³/day were able to remove 92% of COD. Effect of high organic loading rate on the morphology of granules was also reported. Initially at low organic loading rate, granules showed a fluffy morphology inhabited with filamentous bacteria, subsequently transformed into smooth irregular shapes characterized by folds, crevices and depression at high loading rate. These transformations were assumed to permit for better diffusion and penetration of nutrients in to the interior of the granule (Tay et al., 2002a, c; Moy et al., 2002).

2.23.2. Aerobic granules for removal of organics and nitrogen

Successful removal of organics and nitrogen using aerobic granules in SBR were reported. Complete removal of nitrogen involves nitrification and denitrification. The nitrite and nitrate formed by nitrification process are reduced to gaseous nitrogen by denitrifiers (Yang et al., 2004a). Increased substrate Nitrogen/COD ratio support the shift of heterotrophic, nitrifying and denitrifying population within granules. Despite, high Nitrogen/COD ratio increases the nitrification and denitrification it also decreases the heterotrophic population. Apart from Nitrogen and COD, dissolved oxygen also plays a vital role in denitrification process because of certain levels of mixing is necessary for sufficient mass transfer between liquid and granules (Yang et al., 2003b).
2.23.3. Phenolic waste water treatment

Besides nutrient and phosphorous removal, aerobic granules have been used for treating the toxic chemicals like phenol. Fossil fuel refining, pharmaceutical and pesticide processing are the major sources of phenol pollution (Jiang et al., 2004). Possibilities of handling of phenol containing waste water using aerobic granules were reported. Since, aerobic granules are less vulnerable to toxicity of phenol due to their high biomass and much of the biomass not exposed to equal high concentration as present in the waste water they were efficiently used for the treatment of phenol. Using aerobic granular sludge reactor, phenol with 500 ppm was reduced in to 0.2ppm without much deleterious effect of phenol to the granular biomass (Jiang et al., 2004).

2.23.4. Phosphorous removal

Enhanced biological phosphorous removal (EBPR) is a process in which phosphorous has been removed without chemical precipitation. It operated on the basis of combination of both anaerobic and aerobic conditions alternatively in which, substrate feeding is limited to the anaerobic stage. In EBPR, attaining stable and reliable operation was difficult since this method get failed (Barnard et al., 1985; Bitton,1999). To weed out the above said failure, attempts were made with development of phosphorous accumulating microbial granules using SBR and the successful removal of phosphorous evidenced by the granules had phosphorous accumulating characteristics. Result of this study was shown the phosphorous uptake of granules was increased in the range of 1.9% to 9.3% by weight (Lin et al., 2003).
2.23.5. Heavy metal bioremediation using aerobic granules

Aerobic granules are rigid, compactly packed, having large surface area with clear outer surface. Each gram of granule loaded with millions of microbes. Besides it has high porosity, enriched with EPS and other charged moieties. These properties enable them as an ideal biosorbent for heavy metal removal. Aerobic granules are highly tolerant to heavy metals (Xie., 2003). Biosorption of Zn\textsuperscript{2+}, Cd\textsuperscript{2+}, Cu\textsuperscript{2+}, Cr (VI), U (IV) by aerobic granules had been reported several times. It was shown that adsorption of Zn\textsuperscript{2+} was dependent on the initial concentration of Zn\textsuperscript{2+} and concentration of granules. The maximum amount of Zn biosorption by aerobic granules was reported as 270 mg/gm of granules. For Cd\textsuperscript{2+} this adsorption capacity was 566 mg/gm of granules (Liu et al, 2002; 2002c; 2003a; 2003d; Nanchariah et al, 2010; 2006a).

2.23.6. Phototrophic biofilms and phototrophic granules

Heterotrophic granules have been reported to be useful in wide range of applications, ranging from domestic water treatment to bioremediation of hazardous chemicals, heavy metals and reduction of organic compounds. Despite their immense applicability, industrial usage of the heterotrophs are limited, since they need reduced carbon as energy input for biological process which increases the cost of operation. Phototrophic biofilms (cyanobacterial mat and aquatic biofilms) are self sustaining and exists as complex microbial consortia containing several types of phototrophs and heterotrophs. They perform various ecological functions such as primary production, nutrient recycling, pollutant detoxification and biogeochemical cycling (Kumar and
Venugopalan, 2015). Phototrophs in phototrophic biofilm can fix carbon and nitrogen and it could be utilized by the heterotrophs in the biofilm. Diversity of phototrophic biofilms makes them capable of being employed for potential applications in environmental biotechnology. Though the biotechnological potential of phototrophic biofilms is encouraging, their large scale application is difficult due to their need of large area for sufficient irradiation which in turn increases the cost of reactor operation. A simple remedy for this problem was proposed by Kumar and Venugopalan (2015), wherein they developed a technology by combining aerobic microbial granules with phototrophic biofilms. This technology provides solutions for two problems such as high cost input and need of large space for irradiation (Kumar and Venugopalan, 2015).