CHAPTER II

LITERATURE SURVEY

2.1 COPPER

2.1.1 Sources

2.1.1.1 Natural

In nature, Cu is found in many physico-chemical forms, such as, Cu in the forms of sulfides, sulfates, sulpho salts, carbonates and other compounds, for example, chalcite (Cu), chalcocite (Cu_2S), covellite (CuS), bornite (Cu_5FeS_4), chalcopyrite ($CuFeS_2$), cubanite ($CuFe_2S_3$), enargite (Cu_3AsS_4), cuprite (Cu_2O), tenorite (CuO), chalcanthite ($CuSO_4.5H_2O$), brochanite ($Cu_4(OH)_6SO_4$) and azurite ($Cu_3(OH)_2(CO_3)_2$).

Copper is abundant in the lithosphere. An average amount of Cu in the lithosphere is considered to be 70 milligram/kilogram (mg/kg), in the Earth's crust ranges from 24 to 55 mg/kg and in soil is 20-30 mg/kg. In soil, Cu is generally associated with soil organic matter, oxides for iron (Fe) and manganese (Mn), soil silicate clays and other mineral (Baker, 1990). In aquatic environment, natural rock weathering adds about $2x10^5$ tons/year (t/yr.) of Cu into the river systems of the world. Cu concentration in surface water generally rarely rises above 5 microgram/liter (μg/L) or 0.1 micromole, μmol/L, (Spear and Pierce, 1979). In natural water, Cu is wide variety of physico-chemical forms,

many of which are of low bioavailability and/or toxicity. But in acidification of surfaces water will therefore increase the availability of Cu. In water, Cu ions coordinate with water molecules to form aquo ions ($Cu(H_2O_6)^{2+}$. Complexes are formed by successive displacement of water molecules (Taylor et. al., 1996). Furthermore, inputs of atmospheric Cu to soil and aquatic environment by rain and dry deposition can be observed (Taylor et. al., 1996).

2.1.1.2 Man-made

Copper has been contaminated to the environment by human-being in many ways because Cu is advantageous to use in many industrial and agriculture purposes. Cu is one of the major groups of industrially commercial metals which dues to its excellent electrical, thermal conductivity and others. It is extensively used in the manufactures of such equipment, for example, electrical current, electrical contacts, cables and wires, electroplating and alloys, etc. Releasing of Cu to the environment is quite high. Heavy metal-containing sewage sludge (Cu is included) from wastewater treatment is widely used as biofertilizer and soil amendment agent. Otherwise, Cu fume, dust and mist from various industrial exposures increase Cu concentration in the environment.

Copper is an essential element to both plants and animals in trace amount. But in high level, it becomes toxic. Cu is usually used as fertilizer, fungicide, insecticide and algaecide to promote growth of plants, to control fungi, insects and algae, respectively. Those applications bring up copper to the environment.

2.1.2 Physical and Chemical Properties

Cu is reddish colored, take on a bright metallic luster, malleable, ductile and good conductivity of heat and electricity. With atomic number (A.N.) 29, molecular weight (M.W.) 63.54, melting point (M.P.) 1083°C and boiling point (B.P.) 2567°C, Cu is the first element of Subgroup 1B of the Periodic Table. The electronic structure of Cu atom is $1s^2$, $2s^2$, $2p^6$, $3s^2$, $3p^6$, $3d^{10}$, $4s^1$. The single 4s electron is outside the filled 3d shell and is rather stable. Like all elements of the first transition series (e.g. chromium, Cr, cobalt, Co, nickel, Ni, Mn, Fe) and unlike lithium (Li), sodium (Na), potassium (K) and rubidium (Rb), of Group I, two electrons are removed relatively easily from Cu atoms (Parker, 1981). Cu is insoluble in hot or cold water but soluble in nitric acid (HNO_3), hot sulfuric acid (H_2SO_4) and dissolves slowly in ammonia water. In other forms, for example, copper oxide (CuO) has M.W. 79.54 and M.P. 1226°C, insoluble in hot or cold water, soluble in ammonium chloride (NH_4Cl) and potassium cyanide (KCN); hydrated copper acetate ($Cu(C_2H_3O_2)_2.H_2O$) has M.W. 199.65, M.P. 115 and 240°C, soluble 72 g/L in cold water, 200 g/L in hot water and 71.4 g/L in alcohol and copper chloride ($CuCl_2$) has M.W. 134.44 and M.P. 620 ^{0}C, soluble 706 g/L in water (0 ^{0}C), 1079 g/L in water (100 ^{0}C) and 530 g/L in alcohol (15 ^{0}C).

2.1.3 Uses

In industrial application, Cu is one of the major groups of commercial metals. They are widely used because of their excellent electrical and thermal conductivity, outstanding resistance to corrosion and fatigue and good strength. They are generally nonmagnetic. They can be readily soldered and brazed and many copper and copper alloys

can be welded by various gas, arc and resistance methods. For decorative parts, standard alloys having specific color are readily available.

Pure Cu is used extensively for cables and wires, electrical contacts and a wide variety of other parts that are required to pass electrical current. Otherwise, its used for automobile radiators, heat exchanges, home heating systems, panels for absorbing solar energy and other applications requiring of heat across or along a metal section. It is widely used in many forms, for example, copper sulfate ($CuSO_4.5H_2O$) has the greatest amount of uses in agriculture, animal husbandry, steelmaking, treatment of natural asphalt and petroleum industry, copper oxide (CuO) has many uses in catalyst, batteries, desulfurizing oils, paints, insecticides and ceramic colorant, copper acetate ($Cu(C_2H_3O_2)_2.H_2O$) is used in pigment, insecticides, fungicide, mordant and mildew preventive, copper carbonate ($CuCO_3.Cu(OH)_2$) is used in pigments pyrotechnics, insecticide, fungicides and brass coloring, copper cyanide ($Cu(CN)_2$) is used in electroplating of Cu on Fe and copper chloride ($CuCl_2$) has addition uses as a disinfectant, in metallurgy, preservation of wood pulp, deodorizing and desulfurizing petroleum distillates, photography and water purification.

In agricultural, Cu is usually used as fertilizer due to Cu is essential element to plants such as copper acetate ($Cu(C_2H_3O_2)_2.H_2O$), cuprite (Cu_2O), chalcocite (Cu_2S), azurite ($2CuCO_3.Cu(OH)_2$) and chalcanthite ($CuSO_4.5H_2O$) is used to increase crop production. The usual Cu fertilizer source is $CuSO_4.5H_2O$ (bluestone), because hydrated $CuSO_4$ is quite water soluble and is compatible with most fertilizer material, although other compounds, mixtures or chelaters are also used

(Baker, 1990). Otherwise, Cu is used to control insect, algal and fungi as insecticide, algaecide and fungicide. Copper sulfate mixed with lime has been used as a fungicide. In animals, Cu is also taken orally as a diet supplementary in the form of $CuCO_3$ or $CuSO_4$ for administering to pigs and cattles where the solid is deficient in molybdenum. Molybdenum is found in many important enzymes that can be isolated from cow milk. Molybdenum concentrations in animals has a unique relationship to Cu concentration and is referred to as the " Mo-Cu antagonism " (Kendrick., et al,1992).

For medicinal purpose, copper sulfate is used as an emetic, astringent and anthelmintic (Beliles, 1975). Some Cu compounds have shown antitumor and anticancer activity. Kendrick and the colleague (1992) found that Cu^{2+}-bis-thiosemicarbazone, Cu(KTS), one of these drugs was potent activity against tumor (**Figure 2.1**). Copper salts have been used as a food additive to give a bright green color to canned peas, and etc.

2.1.4 Toxicity

2.1.4.1 Man

Cu is an essential trace element for man and has an important role in oxidative enzyme systems, i.e., ascobate oxidase and its relation to ceruloplasmin, cytochrome C oxidase, polyphenol oxidase, amine oxidases and other Cu-protein complexes. Several functions of Cu in those Cu-protein complexes were found, i.e., formation of hemoglobin, collagen and brin muelin, the metabolic pathways related to energy production (Cytochrome C) and tyrosinase activity (Stokinger, 1986).

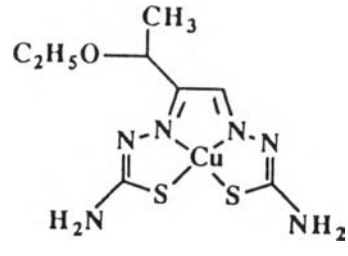

Figure 2.1 Cu^{2+}-bis-thiosemicarbazone, Cu(KTS)

Absorption of Cu, the intestinal mucosa acts to some extent as a barrier to the absorption of ingested Cu. Initially, Cu is bound to serum albumin and later more firmly bound to alpha-ceruloplasmin, where it is changed in the cupric form. Because most cuprous salts are insoluble in water but they tend to oxidize to the cupric forms. The normal serum level of Cu is 120 to 145 μg/L. The bile is the normal excretory pathway and plays a primary role in Cu homeostasis. The storage organs for excess amount of Cu is the liver and bone narrow. While Cu is an essential element, the range between deficiency and toxicity is low in those without effective barriers to control absorption. Man are less sensitive to Cu, because of a better developed homeostatic mechanism. It is believed that excessive Cu exposure in normal persons does not result in chronic disease (Beliles, 1975). These is no increase in Cu tissues storage with age, but serum Cu level does increase. Anyway, Cu may cause of chronic effects in unusual genetic patients. For examples, Wilson's disease, chronic Cu-poisoning, was firstly described by Wilson (Wilson, 1912). The symptom of the disease is: Higher copper accumulating than normal quantity in the liver dues to excessive copper can not be eliminated via to bile and ceruloplasmin that act as transport agents of Cu. So, the liver is saturated with Cu, after that, Cu is infiltrated into the blood stream and binds to important tissues, i.e., brain, cornea and kidney. In the United State studies, Cu levels in various tissues, for example, in the liver, the kidney, the brain, long bone and cornea in normal people are 7.8, 2.8, 5.4, 2.9 and 3.8 microgram/gram (μg/g), respectively. In Wilson's disease, the amounts of Cu in those fresh tissues are remarkably higher, i.e., 99.2, 36.2, 54.9, 31.0 and 35.1 μg/g, respectively. The effect of excessive Cu on those tissues are able to be detected by deformation of the tissues (Stokinger, 1986).

Acute poisoning resulting from ingestion of excessive amounts of oral Cu salts, most frequently copper sulfate, it brings to nausea-vomit, stomachache and diarrhea and may produce death. Otherwise, Cu fume, dusts and mist from industrial exposures affect to upper respiratory tract as metal fume fever and atrophic change in the nasal mucous membranes (Stokinger, 1986).

2.1.4.2 Animals

Essential elements for animals are divided into two groups, i.e., the major elements and the trace elements. The major elements are calcium, phosphorus, magnesium, potassium, sodium, chlorine and sulfur. The trace elements are zinc, iron, manganese, molybdenum, cobalt, iodine, chromium, nickel and copper. Cu is one of the essential elements to the metabolisms of many animals. Cu is important because it participates in many enzymatic reactions and is constituent of the active site of a number of enzyme (Heliovaara and Vaisanen, 1993). Cu deficiency can also result in anemia as Cu is required for the mobilization of iron. Otherwise, Cu deficiency makes some defection in collagen and elastin synthesis (lysyl oxidase) and myelin synthesis (cytocrome oxidase). However, in high quantities, Cu can be toxic in acute toxicity and chronic effect. Cu is one of metals mobilized in the environment by human in high rates. Since it is toxic to organisms Cu has been received considerably attention.

In acute toxicity of Cu, it appears that the most toxic Cu salts are sulfate and chloride about 7 mg/kg intraperitoneally is the LD_{50} for the experimental mouse. Hydrated forms ($CuSO_4.5H_2O$, $CuCl_2.2\ H_2O$) are less toxic (33 and 9.4 mg/kg, respectively) and cupric cyanide has a

lower toxicity. Otherwise, Cu metal dust appears to be the most toxic of all about 3.5 mg/kg intraperitoneal is the LD_{50} for the mouse (Stokinger, 1986). In chronic effects, Cu leads to injury of the liver, kidney and spleens (Boughton and Hardy, 1934). Cu sulfate fed ad libitum in the diet of rats at level of 500 parts per million (ppm) caused delayed growth and 4,000 ppm caused starvation and death (Boyden, Potter and Elvehjem, 1938). Furthermore, excess of Cu storage in the liver of sheep leads to haemolysis and death (Albert, 1985).

2.1.4.3 Plants

Similar to animals, Cu is a trace essential element for plants. It is an essential constituent of many enzymes including phenolase (tyrosinase) which plays an important metabolic role in plants. A bright blue Cu-containing pigment (plastocyanin) is involved in photosynthetic electron transport of plants (Albert, 1985). Cu is used as fertilizer for increased crop production, for example, foliar-applied $CuSO_4.5\ H_2O$ gave a 13.5% increase in wheat yields and 18% increase in sugar beets (Shorrocks and Alleyway, 1987). Cu deficiency is affected to the quality of crop products including size, shape, composition and nutrients value. Some examples of effects of Cu deficiency on some characteristic of plants are: unattractive appearance and smaller size for citrus fruits, discoloration and spongy texture in onions and discoloration in carrots (Baker, 1990). As mention above, Cu is an essential element and Cu deficiency is effects on crop production and others. However, in high quantities or excess Cu can be toxic to plants, for example, delayed growth rate and caused to death, etc.

2.1.4.4 Microorganisms

Similar to animals and plants, microorganisms need both the main and major elements including carbon (C), nitrogen (N), hydrogen (H), oxygen (O), sulfur (S) and phosphorus (P) and the minor or trace elements including iron, Cu, Co, and zinc (Zn). Cu acts as cofactors of different enzymes and other compounds in the cells, but in a very small or trace amount. However, Cu can also be extremely toxic to microorganisms, by impacting microbial growth, morphology and biochemical activities. The effects of Cu on some microorganisms were extensively investigated, especially in bacteria, decreased the growth of ***Escherichia coli*** (***E. coli***, Blundell and Wild, 1969), inhibited the growth of ***Streptococcus faecalis*** (***S. faecalis***, Schoot and Young, 1973), and inhibited the rate of respiration of ***Bacillus megaterium*** (***B. megaterium***, Cassity and Kolodziej, 1984). In algae, 0.005 ppm of Cu reduced 60% of the growth of ***Spirulina platensis*** (Kallqvist and Meadows, 1978); inhibited the growth of the green algae ***Scenedesmus obliquus*** **LH** (Drbal, Veber and Zahradnik,1985); Cu, Zn and Pb (Lead) were toxic to the growth of freshwater green algae, ***Scenedesmus quactricanda***; the sequence of toxicity was Cu > Zn > Pb (Starodub, Wong and Mayfield, 1987); and inhibited the photosynthesis in ***S. platensis*** (Kallquist and Meadow, 1978).

2.2 METHODS OF METAL REMOVAL

2.2.1 Physico-Chemical Methods

2.2.1.1 Activated Carbon

Activated carbon adsorption is a popular method in both water and wastewater treatment to remove soluble heavy metal ions, for

example, the removal of copper and chromium from electroplating wastewaters (Smithson, 1971).

Activated carbon, amorphous form of carbon, is made from different raw materials, i.e., coal, wood, coconut shells, pulp mill residues, petroleum base residues and char from sewage sludge pyrolysis. It is sequential produced by controlled dehydration, carbonation and oxidation. Activated carbon is not a pure carbon and has high affinity to adsorb heavy metals and other compounds. It is able to be used in either granular or powder forms, depending on the application and economic (Wentz, 1995).

Adsorption on activated carbon occurs when it contacts to solution and will be stopped at the rate of adsorption and desorption is equal. The capacity of adsorption per unit weight of carbon is as increase as the concentration of solute is increased. Adsorption equilibrium is controlled by two types of interaction, i.e., solute-adsorbent and solute-solvent. There is competition between the forces of dissolution and adsorption, any charge in a system which tends to decrease the dissolution force or increase the adsorption forces will shift equilibrium toward higher adsorption per unit weight of carbon. Anyway, adsorption is depended on various factors, i.e., surface area, pore sizes, solubility of solute, pH and temperature.

2.2.1.2 Precipitation

The usual method for removal of heavy metals is chemical precipitation. Precipitation have been used to removal of metal from wastewater, including Cr, Cu, Zn, Pb, Cd (cadmium), Mg (magnesium)

and Hg (mercury). Precipitation of heavy metal is depended on pH. Due to the type of metal ion has resulting in the formation of an insoluble form.

The hydroxide of heavy metals are usual insoluble, generally, lime is used to precipitate them. Precipitation of heavy metal is depended on pH. The optimum pH is unique for each metal. Furthermore, carbonates and sulfides are used to precipitate heavy metal. By addition of sulfide chemical, such as sodium sulfide (Na_2S) or sodium bisulfide (NaSH). The addition of sulfide compounds must be controlled to minimize odor and potential toxicity, because precipitation by sulfide is able to generate another unattractive by-product, hydrogen sulfide (H_2S) gas.

2.2.1.3 Evaporation

Evaporation of liquid is applied in heavy metals management. This method is depended on a vapor pressure. At high vapor pressure, evaporation will be readily, but at low vapor pressure, evaporation is more slowly. The boiling temperature of a pure liquid has been reached when the vapor pressure of liquid equals the atmospheric pressure. Soluble salts and other compounds elevate the boiling point. So, physical separation techniques should be used before evaporation process.

Evaporation are used in single and multi-effect evaporation. To conserve energy and enhance separation of volatile wastes from liquids, a multi-effect evaporator may be used.

2.2.1.4 Reverse Osmosis

Osmosis is a process which a solvents flows through a semipermeable membrane from a dilute to a more concentrated solution (Hess, 1988). In general, the solvent flows in the direction that will reduce the concentration of solution. The osmotic pressure of the solution is applied to the solution will just prevent the passage of the solvent through the semipermeable membrane. In reverse osmosis, a differential pressure that exceeds the osmotic pressure is applied to the membrane, causing the solvent to flow from the stronger to the weaker solution. In application, reverse osmosis is used to removal heavy metal include Cu from electroplating rinse water and desalination of sea or blackish water.

2.2.1.5 Ion Exchange

Ion exchange is a reversible exchange of ions between liquid and solid phases. Ions held by electrostatic forces to charge functional groups on the surface of an insoluble solid are replaced by similar ions charge in a solution (Wentz, 1995). Ion exchange materials should have ion active site in whole structure, high capacity, selectivity for ionic species, capability of regeneration, chemical and physical stability and low solubility.

Synthetic ion exchange material is widely used in commercial application, its has both cation and anion exchanges, cation exchange resin has a high ion exchange capacity and has reactive groups, such as sulfonic, phenolic and carboxylic group and anion exchange resin has reactive groups, such as quaternary ammonium or amine groups.

2.2.1.6 Conclusion

The primary sources of Cu in industrial wastewater are metal-process picking baths and plating baths. Cu may also be present in wastewater from a variety of chemical manufacturing processes employing copper salts or copper catalyst. Cu is removed from wastewater by precipitation, ion exchange, evaporation, electrodialysis and activated carbon. Ion exchange or activated carbon are feasible treatment methods for wastewater containing less than 200 mg/L Cu. Copper is precipitated as a relatively insoluble metal hydroxide at alkaline pH. In the presence of high sulfates, calcium sulfate will also be precipitated that will interfere with the recovery value of the copper sludge. This may dictate the use of a more expensive alkali such as NaOH to obtain pure sludge. Cupric oxide has a minimum solubility between pH 9.0 and 10.3 with reported solubility of 0.01 mg/L. Field practice has indicated that the maximum technically feasible treatment level for copper by chemical precipitation is 0.02 to 0.07 mg/L as soluble copper. Precipitation with sulfide at pH 8.5 will result in effluent copper concentration of 0.01 to 0.02 mg/L. Low residual concentration of copper are difficult to achieve in the presence of complexing agents such as cyanide and ammonia. Removal of the complexing agent by pretreatment is essential for high copper removal. Copper cyanide ($Cu(CN)_2$) is effectively removed by activated carbon (Sittig, 1977).

2.2.2 Biological Methods

2.2.2.1 Activated Sludge

In biological methods, activated sludge systemis widely used to remove organic waste from wastewater. This process is a biological wastewater treatment technique in which a mixture of wastewater and

biological sludge in the presence of oxygen. In practice, wastewater flow into aeration tank where air is injected to mix the activated sludge with the wastewater and to supply the oxygen for the organisms to break down the organics. The mixture of activated sludge and wastewater in the aeration tank is called mix liquor. The mix liquor flows from the aeration tank to a secondary clarifier where the activated sludge is settled out. The settled sludge is return to the aeration tank (return sludge) to maintain microbes to breakdown the organic.

Organics which can be decomposed by aerobic microorganisms include polysaccharide, protein, fat, alcohol, aldehyde, fatty acids, alkenes, cycloalkanes and aromatic. But, isoalkanes, halogenated hydrocarbons and lignin are more resistant to microbial decomposition, however, decomposition does occur at slow rate (Dugan, 1972). However, not all of organic removed from the wastewater influent are decomposed, for example, polychlorinated biphenyl (PCB, Dugan, 1972).

Metals present in concentration which is non toxic to bacteria in activated sludge systems are removed by the treatment process. Insoluble metal, such as, metal oxides and hydroxide are removed by sedimentation before activated sludge treatment. Soluble metals and fine metal particulate are concentrated in the biomass, primary through adsorption onto the activated sludge surface (Oliver and Cosgrove, 1974; Esmond and Petrasek, 1974). It suggested that the biomass surface is coated with a polysaccharide which consist of glucuronic acid and neutral sugar. Metal ions form salts with the carboxyl groups present in the exopolysaccharide coating and are electrostatically attached to the hydroxyl group (Steiner, McLaren and Forster, 1976). In a study of

Cheng, Patterson and Miner (1975) which studied heavy metal uptake by activated sludge, found that in an activated sludge effluent containing 2100-25,200 μg Cu/L, i.e., 89% of total amount of Cu was removed during treatment. Furthermore, 98% of the lead was removed from effluent containing 2100-25,500 μg Pb/L. In another study has shown that in 900 μg Zn/L containing effluent, 89% of Zn was removed during sewage treatment (Barth et al., 1965).

The removal of heavy metals from wastewater and sewage relies primary on the immobilization and complexation of metals by extracellular compounds, for example, exopolysaccharide or EPS (Brown and Lester, 1982 and Rudd et al., 1984) as mention above. The genus **Zoogloea**, an important bacterium in sewage treatment, readily forms an ionic slime matrix. Toxic metals complex with the matrix and precipitate out of solution. ***Klebsiella aerogenes*** is another common sewage bacterium which binds metal ion with EPS. Complexed metal are then removed from the wastewater via sedimentation during the treatment process.

Other mechanisms of metal removal from sewage include physical capture by microbial flocs, cellular accumulation and volatilization by such organisms, i.e., ***Klebsiella***, ***Pseudomonas***, ***Zoogloea*** and ***Penicillium*** **sp.** (Brown and Lester, 1979).

2.2.2.2 Aerated Lagoons

The technique of aerated lagoons developed from adding artificial aeration to existing waste stabilization ponds. The aerated lagoon process employs aeration equipment same in the activated sludge

treatment process. The process has been called a "dilute activated sludge" treatment process. The major difference with aerated lagoon is that microorganisms are not continuously circulated from final clarifiers back to the head of process.

Because aerated lagoons usually are not as well mixed as activated sludge process, a low level of suspended solid is maintain in the process mixed liquor. Mixed liquor volatile suspended solids (MLVSS) of aerated lagoon generally range from 50 to 150 mg/L (Eckenfelder, 1970). However, lower level of mixing and aerating can be increased to level comparable to activated sludge treatment so that higher MLVSS loading can be supported.

The most important modification of aerated lagoon occurs when aeration is not sufficient to maintain aerobic condition. A portion of suspended solids including biomass is settle to the bottom and undergo anaerobic microbial decomposition. Lagoon having the characteristic are called facultative lagoons. At the settled-sludge facultative anaerobic bacteria decompose simple organic such as carbohydrates and protein to volatile organic acids.

The aerated lagoons requires retention times, that longer than activated sludge treatment. For facultative anaerobic lagoon, longer retention time are necessary to support the slower metabolic rate of anaerobic microorganisms. Anaerobic lagoons may require up to two days retention, while facultative anaerobic lagoons may need more than four days of retention time.

For the treatment of industrial wastes, it may be necessary to line the basin. Recent lining for waste lagoons consist of pigment-filled polyethylene or polypropylene. For example, Hypalon^{R} is excellent except for some solvents, ketones and chlorinated solvents. The necessity for lining depend on the composition of the influent waste and the in situ soil permeability (Davis and Cornwell, 1991).

2.2.2.3 Trickling Filter

The trickling filter process, wastewater is sprayed though the air to adsorb oxygen and allowed to trickle though a bed of rock or synthetic media coated with a slime of microbial growth. The microbial slime is able to decompose organic matter in the waste steam. Process modification employs various media and depth of media to retain the microorganisms under varying hydraulic conditions.

The trickling filter process relies on media support of immobile microorganisms which receives their organic substrate as waste is trickled over their cell surface. The primary metabolic processes are aerobic and the systems utilize the same type of aerobic bacteria as the activated sludge system. The aerobic microorganisms produce enzymes which perform oxidation and hydrolysis catalysis for the decomposition of simple and complex organic. The microbial slime which coats the trickling media remains aerobic primary at its surface where air and water interface with the cells. The underlying portion, adjacent to the media, may become anaerobic. Decomposition of organic are hydrolyzed to organic acids and its turn are oxidized to methane and carbon dioxide.

Trickling filters decompose all types of organic, as does activated sludge treatment. However, the percentage of removal of organic is lower than activated sludge. Because of the relatively short residence time of wastewater contact with microorganisms.

2.2.2.4 Stabilization Ponds

Waste stabilized ponds are large, shallow basins which provide aerobic and facultative anaerobic decomposition of organic. The ponds rely on long retention periods and natural aeration for the microorganisms to decompose organic to carbon dioxide and water. Natural aeration is encouraged by wind action and algal photosynthesis. The facultative anaerobic decomposition which sometime occurs in the ponds take place at the benthic sediment-water interface.

Waste stabilization ponds support aerobic and facultative anaerobic microorganisms, as well as algae. There is symbiotic relationship between the aerobic microorganisms and the algae. Algae synthesize new cells by using the sunlight as energy source, and carbondioxide and amine as food or carbon sources. The algae provide dissolved oxygen for the respiration of aerobic bacteria, then aerobic bacteria hydrolyze and oxidize organic to carbondioxide and amine by-products.

Waste stabilization pond activity depends on temperature. During warm season, the symbiotic relationship between the aerobic bacteria and algae is most efficient than other seasons.

Metal concentration appears to be more significant in stabilization ponds than in other biological treatment processes. Because metal inhibition is evidenced by decreased respiration of the mixed microbial population and results in lower biochemical oxygen demand (BOD) removal efficiencies. The concentrations of copper and chromium that affect the activities of organisms in the pond are ≥ 0.25 and > 50 mg/l, respectively (Azad and King, 1965).

2.3 MECHANISMS OF COPPER RESISTANCE IN MICROORGANISMS

2.3.1 Bacteria

2.3.1.1 Extracellular Precipitation

Many bacteria are able to produce metabolic products that binds to metal ions. One of the best example of extracellular precipitation of metals is production of hydrogen sulfide (H_2S) by sulfate-reducing bacteria, and H_2S further reacts to soluble heavy metal ions to from insoluble metal sulfide such as covellite (CuS) and sphalerite (ZnS, Ehrlich, 1984). In case of ***K. aerogenes***, lead, mercury or cadmium were precipitated to be insoluble sulfide on the outer surface of those bacterial cells (Gadd, 1990). ***Thiobacillus*** **sp.** precipitates silver (Ag) to silver sulfide; Ag_2S, (Pooley, 1982), and also ***Desulphovibrio*** **sp.** can precipitate a large amount of metals (Kelly, Norris and Brierley, 1979).

2.3.1.2 Binding to Cell Wall

Some bacteria can accumulate heavy metals by binding the metals onto their cell walls. Those bacterial cell walls are anionic because of the presence of carboxyl, hydroxyl, phosphoryl and other negative charged sites. In gram-positive bacteria, most cell walls of them

contain large amounts of peptidoglycan and anionic polymers, e.g., tiechonic or teichuronic acids. Cell walls of ***Bacillus subtilis*** are complex polyanion that are likely sites for accumulation of metal cations. Carboxyl groups are major sites for metal accumulation and teichonic acids involved in metal uptake function in ***B. subtilis*** (Beveridge and Murray, 1980).

Cell walls of gram-negative bacteria are composed of phospholipids, lipopolysaccharide (LPS) and proteins, usually containing 20-25%, 30% and 45-50% by weight, respectively. Cell wall of ***E. coli*** also exhibited metal binding capacity, metal deposition occurred at polar head group regions of constituent membranes or along peptidoglycan layer (Berveridge and Koval, 1981). It appeared that the phosphoryl groups on LPS component of the cell wall was the site for binding of divalent cations, not to free carboxyl groups (Ferris and Beveridge, 1986).

2.3.1.3 Intracellular Accumulation

Bacterial cells can accumulate intracellularly both essential metals (Ca, K, Na, Fe, Mg and Cu) and nonmetabolic metals (Ni, Cd and Co). Intracellular accumulation is an energy-dependent process which require active respiration by the bacterial cell. Active uptake requires a specific transport system. Bacteria have well developed transport system capable of accumulation metals against a concentration gradient. When metal ions were taken into the cell, ions of equivalent charge are released by the cell (Brierley Kelly, Seal and Best, 1985). The pH and other ions can affect intracellular uptake. Uptake of specific metals occurs at certain optimum pH, e.g., Ni uptake in ***Neurospora crassa*** occurs at pH 4

and Zn uptake in ***Neocosmospora vasinfecta*** occurs at pH 6.5 (Gadd, 1986).

2.3.1.4 Binding to Exopolysaccharide

Many bacteria produce large amount of exopolysaccharides that act as efficiently metal biosorbents. The composition of those exopolysaccharide from bacteria isolated from a variety of natural habitats are uronic acid and other substituted sugars (Geesey and Jang, 1989). It was found that the bacterial EPS contains 5-25% uronic acids (Sutherland, 1980), these acids confer a negative charge. However, sialic acids are also found in the EPS in several genera of bacteria. These acids contain free carboxyl groups that confer a net negative charges on the polymer (Geesey and Jang, 1989). Greater detail of EPS will be discussed in Section 2.4.

2.3.2 Fungi

2.3.2.1 Extracellular Precipitation

Fungi detoxify heavy metals by producing of organic metabolites that those properties are chelating or complexation. Most of them are able to form complexes or precipitate heavy metals. Citric acid, for example, is one of an efficient metal-ion chelator, an another is oxalic acid which can interact to metal ions to form insoluble oxalate crystals around cell walls and in the external medium (Murphy and Levey, 1983; Sutter, Jone and Walchli, 1983). The production of H_2S by some yeasts leads to extensive precipitation of metals as insoluble sulfide in and around cell walls (Ashida, Higashi and Kikuchi, 1963; Minney and Quirk, 1985). ***Saccharomyces cerevisiae*** can also precipitate metals as

sulfides in and around cell walls and colonies may appear dark brown in the presence of Cu (Ashida, Higashi and Kikuchi, 1963).

2.3.2.2 Intracellular Accumulation

In intracellular uptake, metal ions are transported into cells across the cell membrane, may be a slower process than the adsorption (Norris and Kelly, 1977, 1979; Borst-Pauwels, 1981). In certain fungi, especially yeast, much greater amounts of metal may be accumulated by such process than by metabolism-independent accumulation (Norris and Kelly, 1977, 1979). Many metals are essential for growth and metabolism, for example, Cu, Fe, Zn, Co and Mn, all organisms therefore have the ability to accumulate these metals intracellularly from low external concentration.

Other anions and cations, pH and organic materials can effect intracellular uptake. In general, rates of intracellular uptake are decreased at low pH values. In growing fungal cultures, phase of adsorption and intracellular uptake may be affected by changes in the physiology and morphology of the fungal and physical and chemical properties of the growth medium (Gadd, 1986). During the lag period or early stage of growth, maximum uptake can be found and was reduced in the stationary phase. This has been shown for Cu in ***Aspergillus pullulans*** (Gadd and Giffiths, 1980), ***Decaryomyces hansenii*** (Wakatsuki et. al., 1979), ***Penicillium spinulosum***, ***A. niger*** and ***Thiobacillus viride*** (Townsley and Ross, 1985, 1986). In ***A. pullulans***, Cu uptake was changed when the pH of the medium was decreased. This evidence occurred during growth and alleviation of Cu toxicity at low pH (Gadd and Giffiths, 1980). However, for several filamentous fungi, pH

decreasing was not only factor for reducing of Cu uptake. A reduction could occur before any significant drop in the pH and also in media where the pH was maintained at 5.5. Cell wall composition during growth and/or the release of metabolites can control metal availability (Townsley and Ross, 1985, 1986).

A common response to metal exposure is the induction of intracellular low-molecular-weight cysteine-rich, metal-binding proteins, called metallothioneins, that have functions in detoxification, the storage and the regulation of intracellular metal ions. The Cu-induced metallothionein of ***S. cerevisiae*** has received the most attention, and there is a recent review dealing with the structure of the CUP1 locus, mechanisms of gene amplification and relationship with resistance. This protein of ***S. cerevisiae*** is induced only by Cu and not Cd or Zn (Butt and Ecker, 1987). Cu-inducible metallothionein has also been documented in ***N. crassa*** (Munger et. al., 1985), whereas in ***Dactylium dendroide***, two Cu-binding proteins were induced but only one was rich in cysteine (Shatzman and Kosman, 1979).

2.3.2.3 Binding To Cell Wall

Fungal can accumulate metals by binding them onto their cell walls. Similar to bacteria, fungal cell walls are anionic owing to the negative charge sites. Many compounds are found in fungal cell walls, i.e., mannans, glucans, phosphate and chitosan phosphate. These compounds can adsorb copper, cadmium, manganese (Mn), uranium (U), zinc, cobalt, nickel, magnesium (Mg) and calcium (Sakaguchi and Nakajima, 1982). It was detected that there are phenolic polymers and melanin in fungi, and also other components, i.e., phenolic units,

peptides, carbohydrates, aliphatic hydrocarbons and fatty acid. They contain various functional groups that are involved in metal bindings to form metal-organic complexes, i.e., carboxyl, phenolic and alcoholic hydroxyl, carbonyl and methoxyl groups, for example, Fe- and Cu-fungal phenolic polymer complexes (Saiz-Jimenez and Shafizadeh, 1984). For fungal phenolic polymers and melanin, the order of adsorption capabilities were Cu > Ca > Mg > Zn (Zunino and Martin, 1977). Hydrolyzed polymers of ***Epicoccum purpurascens*** removed 72% of copper ions in $0.5x10^{-4}$ M solution (Saiz-jimenez and Shafizadeh, 1984).

2.3.3 Algae

2.3.3.1 Binding To Exopolysaccharide

Production of extracellular polysaccharides has been detected in several classes of algae (Kaplan, Christiaen and Arad, 1987). These compounds act as natural metal chelators and thus reduce metal toxicity (Crist et. al., 1981; Laube et. al., 1980). Though, not all the polysaccharides excreted by algae exhibit metal-complexing capacity but a correlation between highly anionic-charged polymers and metal-complexing capacity was found. The amounts of negative charges on the polymer are related to the quantities of uronic acids, which contain free carboxyl groups (Manzini et al., 1984).

The dissolved polysaccharides of ***Chlorella stigmatophora*** were found to be capable of binding to some metal ions, i.e., Cd, Zn, Cu, but not to Pb. The amount of bound metal also depends on the concentration of polysaccharide in the solution. The complexing capability of cupric ions are about 160, 325, 510 and more than 1,000

parts per billion (ppb) in 10, 15, 30 and 45 microgram/milliliter (μg/ml), respectively (Kaphan, Christiaen and Arad, 1987).

2.3.3.2 Binding To Cell Wall

Algal cell walls are similar to bacterial and fungal cell walls that are able to bind to heavy metals. Because of the presence of carboxyl, amide, hydroxyl, phosphate, amino, imdazole, thiol and thioether moieties, those are presented in the proteins, carbohydrates and lipids (Brierley, Brierley and Davidson, 1989). ***Chlorella vulgaris*** and ***C. reguloris*** are studied more extensive because they are quite easy to maintain, cultivate and handling of the organisms. Freeze-dried ***Chlorella*** **sp.** exhibit some certain degree of selectivity of metal ions from the aqueous solutions of those multi-components (at pH 5 and 1 mmol, of each type of ions). In decreasing order, metals are bound selectively as shown, i.e.,

$$UO_2^{2+} > Cu^{2+} > Zn^{2+} > Ba^{2+} = Mn^{2+} > Cd^{2+} = Sr^{2+}$$

At the pH ≥ 5, cupric ions were bound strongly and at the pH ≤ 2, they are eluted from **Chlorella** cell walls (Darnall et. al., 1986). In 1980, Christ et. al. reported that the amount of Cu had been uptaked 0.6 μmol/mg dried algal cell walls. And also, Vancheria, Wehrheim and Wettern (1994) found that Cu had been accumulated 0.58 μmol /mg dried weight of ***C. fusca*** cell wall extract.

2.4 BACTERIAL EXOPOLYSACCHARIDE

2.4.1 Introduction

As mention above, the elaboration of exopolysaccharides in nature are found in a numerous microorganisms. The exopolysaccharide serve as a buffer zone between cells and the surrounding environment. They also act as a barrier to toxic substances, i.e., bacteriophage, antibiotics, biocides and heavy metals (Gadd, 1992). Many of them carry charges which promote ionic and electrostatic binding of counterions. These interactions may prevent excess quantities of charged molecules, e.g., heavy metals from the surrounding. EPS may extend from 0.1 to 10 μm or more from the cell surface into the surrounding environment (Geesey and Jang, 1989).

Natural resistance or tolerance in microorganisms has long been known involving extracellular polymer or exopolysaccharide. In sewage treatment, the floc-forming ***Z. ramigera*** **strain 115** was found to remove significantly more dissolved heavy metals (Cu, Co, Fe and Ni) from the solution than the strain which did not produce EPS around the cells (Brierley, Brierley and Davidson, 1989; Roane, Pepper and Miller, 1996).

2.4.2 Exopolysaccharide Structure

The polysaccharide can be divided into, 2 types, i.e., homopolysaccharide and heteropolysaccharide (Sutherland, 1990). The homopolysaccharide structures are linear neutral molecules composed of a single linkage type. There are also several polyanionic homopolymers, for example, bacterial sialic acid (**Figure 2.2**). The heteropolysaccharide are almost all composed of repeating units varying in size from disaccharide to octasaccharides. These frequently contain one mole of a

uronic acid, which is usually D–glucuronic acid. Very occasionally, two uronic acids are present. The uniformity of the repeat units is based on chemical activities and it is possible that some irregularities may be found, especially in the polymers composed of larger and more complex repeat units. The heteropolysaccharide commonly posses short side-chains, which may vary from one to four sugar moiety in length. Very rarely, the side–chains may also be branched. It is also possible to find exopolysaccharides that contain several different side–chain, usually in the form of single monosaccharide attached to adjacent main chain residue. Bacterial alginates (**Figure 2.3**), one of heteropolysaccharides of irregular structure, are exceptional (Sutherland, 1990). The types of exopolysaccharide structure were summarized in **Table 2.1**.

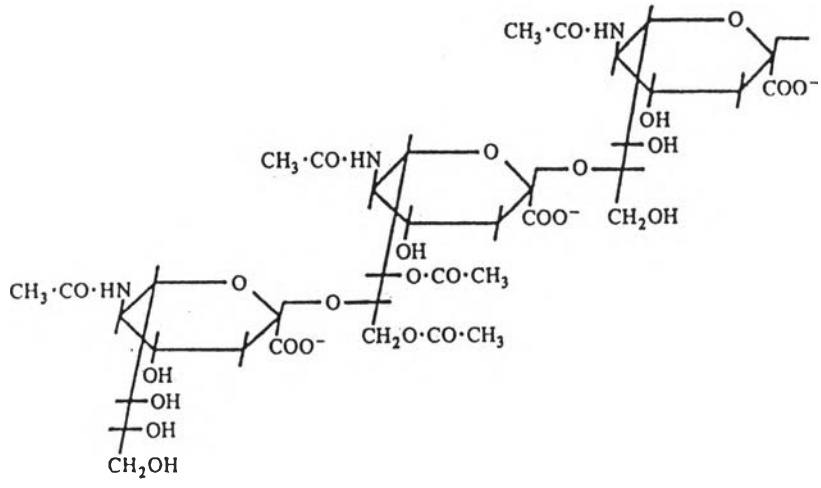

Figure 2.2 The structure of a bacterial sialic acid, the K1 antigen of ***E. coli*** (Sutherland, 1990)

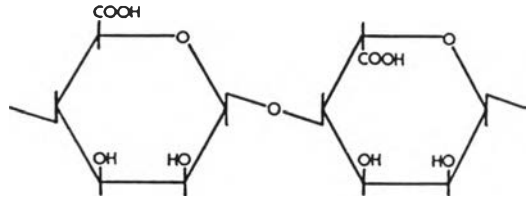

Figure 2.3 Structure of alginate of ***Pseudomonas*** **sp.**
(Devault et.al., 1989)

Table 2.1 Some Structures of Exopolysaccharide were shown (Sutherland, 1990).

Exopolysaccharide Structures	
Homopolysaccharide	Heteropolysaccharide
1. β - D – glucans • Cellulose • Curdlan • Seleroglucan 2. α - D – glucans • Dextrans • Elsinan • Pullulan 3. Sialic acid	1. Bacterial alginates 2. Emulsan and related polysaccharide 3. Gellan and related polymers 4. Hyaluronic acid and heparin 5. Rhizobium heteroglycans 6. Succinoglycan 7. Xantan 8. XM6 9. Agrobacterium, Rhizobium and Zoogloea polysaccharide and related polymers

2.4.3 Genetics

2.4.3.1 Genetic and Regulation of Exopolysaccharide Synthesis

Genetic control of exopolysaccharide synthesis, in ***E. coli*** was studied (Echarti et. al., 1983 and Robert et. al., 1988). It was found that gene cluster controlling exopolysaccharide or K antigens synthesis. There were various types of K antigens including K 1 serotype composed of sialic acid. A region of DNA, approximate 17 kilobase (kb), formed at least three functional segments. The first part, about 9 kb, appeared to code for gene functioning in the translocation of the completed polysaccharide to the bacteria surface. The second part, about 5 kb, consisted of the genes responsible for the synthesis of the enzyme groups involving in biosynthesis of the sugar nucleotides specific to the polysaccharide, as well as specific transferases and polymerases. Mutation in the second part caused polysaccharide synthesis failure. The function of the third part was clearly defined, being apparently involved in modification of the exopolysaccharide after it had reached the cell surface. It might include attachment of the polymer to the cell surface through a terminal linkage including the sugar ketodeoxyoctonic acid (Echarti et. al., 1983). Transposon mutagenesis in this region caused intracellular accumulation of the polysaccharide in a form differing from the mature polymer. Mutation in region 1 and 3 prevent expression of exopolysaccharide at the bacterial surface but do not inhibit the enzymatic reactions of polymer synthesis. On the other hand, one phenotype, observed from mutants in region 2, is the absence of polysaccharide production in *vivo*. Analysis of the genes of region 1 in different ***E. coli*** strains indicated DNA sequence homology; analysis of the protein encoded by the homologous DNA sequence revealed set of

similar polypeptides. Complementation of the function in region 1 and 3 was possible, indicating that modification of exopolysaccharide after polymerization and transport of the mature polysaccharide from the site of synthesis, are mechanisms common to the different ***E. coli*** strains and are independent of polysaccharide structure. Proposed genetic regulation of exopolysaccharide synthesis in ***E. coli*** was shown in **Figure 2.4** (Robert et. al., 1988).

The central cassette of gene responsible for biosynthesis is unique to each bacterium and varies in sequence and size depending on the size of the repeat unit and the quantity of genetic information required. In other polysaccharide-synthesizing bacteria, the genetic organization may be different; this type of genetic control may be limited to certain type of capsular material formed by ***E. coli*** strain or to species of **Enterobacteriaceae**. Recently, some homology has been demonstrated between genes involved in polysaccharide synthesis in ***E. coli*** and ***K. aerogenes***.

Both of ***Agrobacterium tumefaciens*** and ***Rhizobium meliloti*** are capable of synthesizing succinoglycan, some common features might be expected in the genetic control of this exopolysaccharide. In ***R. meliloti***, three loci involved in polysaccharide production (*exoA*, *exoB*, *exoF*) were carried on a plasmid. In ***A. tumefaciens***, all the loci identified as having roles in exopolysaccharide synthesis appear to be chromosomal. Curing of the Ti plasmid in ***A. tumefaciens*** leaves the cell exo^{+}. ExoC mutants from each bacterial species were similar, each being pleiotropic, slow growing and partially defective in exopolysaccharide synthesis; the locus may code for a cell surface structure affecting

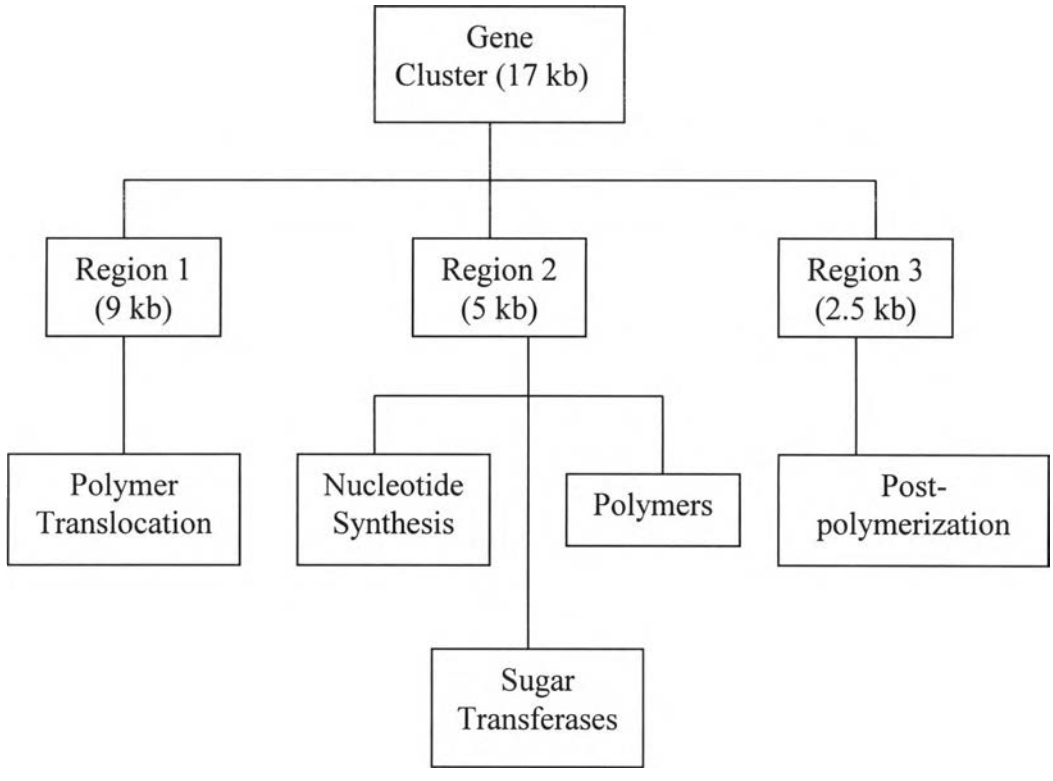

Figure 2.4 The cassette of ***E. coli*** genes thought to control polysaccharide synthesis
(Source; Robert et. al., 1988).

surface-associated phenotypes. Despite the difference in the genotype distribution of the loci concerned with exopolysaccharide synthesis in the two genera, analogous complementation groups were found in any non-exopolysaccharide-forming mutants (Sutherland, 1990).

The specific system of genetic control in polysaccharide synthesis, for instance, the genetic of colanic acid synthesis in ***E. coli*** and other **Enterobacteriaceae** that produce colanic acid, the synthetic process appear to be complex regulatory system. Four loci are now known to function in this *rcsA* and *rcsB*, as positive regulators but Ion and *rcsC*, as negative regulators. The three *rcs* loci map at 43, 47 and 48 minutes, respectively, on the ***E. coli*** chromosome. The Ion locus at 63 minutes is in concern with overproduction of exopolysaccharide. This locus is linked to *serA* and *kps* genes involved in the synthesis of several ***E. coli*** capsular (K) antigen (Sutherland, 1990).

Alginate synthesis in ***Pseudomonas aeruginosa***, four genes (A-D) in alginate control synthesis control the enzymes yielding the activated precursor GDP-mannuronic acid (**Table 2.2**). Most mutants affecting alginate production are located at 45 minutes on the chromosome, near the *argF* gene. Exception the *algB* gene in the 21 minutes region, *algR* is closed to *argH* at 19 minutes (Sutherland, 1990).

Table 2.2 Genes involved in alginate synthesis in ***P. aeruginosa*** (Sutherland, 1990).

Gene	Enzyme(s)
Alg A	Phosphomannose isomerase
Alg B	phosphomannomutase
Alg C	GDP-mannose pyrophosphorylase
Alg D	GDP-mannose dehydrogenase

2.4.3.2 Genetic of Copper Resistance

Many bacterial strains contain genetic determinants of heavy metal resistance, such as, Ag^{+}, AsO_2^{-}, AsO_4^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Sb^{3+}, TeO_3^{2-}, Ti^{+} and Zn^{2+} (Silver and Phung, 1996). These resistance systems have been found on plasmid and transposons (Silver, 1981; Silver and Misra, 1984 and Summers, 1985), but, frequently, chromosomal genes in other organisms determine related systems. In addition, bacterial chromosomes contain genes for resistance to many of the same heavy metal cations and oxyanions, as do plasmids (Silver and Phung, 1996).

Bacterial resistance to copper conferred by plasmids have been reported in **Pseudomonas** (Cooksey, 1994), **Xanthomonas** (Lee, Hendson and Panopoulus, 1994) and ***E. coli*** (Brown et. al., 1995; Brown, Lee and Silver, 1994). These systems are highly homologous (Brown, Lee and Silver, 1994; Cooksey, 1993, 1994) and contain the same genes (Silver and Phung, 1996). For instances, ***Pseudomonas* sp.**, the two regulatory genes are called *copR* and *copS* and the four structural genes *copABCD*.

The best understood copper transport and resistance systems today is that of the gram-positive pathogen ***Enterococcus hirae*** (***S. faecalis***); (Solioz, Odermatt and Krapf, 1994). The copper transport ATPases and metallothionein cation-binding proteins are only known from chromosomal genes. The two genes, *copA* and *copB* that determine, respectively, uptake and efflux p-type ATPases are found in a single operon (Odermatt et. al., 1993).

The detailed mechanisms for copper-resistance system (Influx-Efflux) have been studied in many bacterial strains, but today the detailed mechanism with regards to genetic control to copper-resistance that related to exopolysaccharide is not published today. Thus, although bacterial copper resistance is widespread, it is only beginning to be studied.

2.4.3.3 Genetic Strategies for Strain Improvement

A) Genetic strategies for improvement the synthesis of exopolysaccharide.

It has long been known that microorganisms usually involve the removal of metals. Biologically synthesized exopolysaccharide can be used to treat heavy metal. All of the various process used by microorganisms to interact with metals may be amenable to improved performance through genetic manipulation. In addition, microbial synthesis and elaboration of complex molecules such as polysaccharide can remove metal by chelation to this exopolysaccharide. The synthesis and binding properties of these polymers can also be improved through genetic manipulation (Ensley, 1994).

The exopolysaccharide of both gram positive and gram-negative bacteria bind a variety of toxic heavy metal (McLean and Beveridge, 1990). While heavy metals can be concentrated out of solution on to cell walls, membrane and exopolysaccharide, genetic change to improve the performance of metal binding by bacteria cannot be given today's technology. However, genetic changes in the synthesis of exopolysaccharide may be useful in improving heavy metal binding by bacteria (Ensley, 1994).

The synthesis of exopolysaccharide can be dependent upon external conditions and the presence of the specific pathways for carbohydrate synthesis, manipulation of growth conditions or the use of mutagenesis or genetic engineering techniques could improve the biological absorption of metals from aqueous environments. Studies describing the cloning, artificial regulation or over-expression genes involved in exopolysaccharide biosynthesis have not yet been published. When these methods are developed, synthesis of metal binding polymer by microorganisms could markedly improve economics of biosorption and expand the range of application that are competitive with chemically synthesized polymers and other metal removal methods (Ensley, 1994).

B) Genetic strategies for improvement the structure of exopolysaccharide

Exopolysaccharide elaborated by microorganisms can take many forms. Factors controlling the structure of exopolysaccharides are not well understood and these molecules are not readily altered using current genetic techniques. Genetic changes that affect the structure of exopolysaccharides, while more difficult to perform, promise to yield substantial benefit in increasing the efficiency of metal binding by microorganisms. Changes in the composition of exopolysaccharide caused by manipulating culture conditions suggest that engineering of biosynthetic pathways can also be used to affect the density of acidic groups on exopolysaccharide. Once the genes involved in controlling the structure or composition of acidic side-chains have been identified, alteration in the regulation or expression level of these genes could result in the synthesis of new polymeric materials with higher binding capacities or lower binding constant. While random mutagenesis and

selection techniques may be successful in improving heavy metal binding by exopolymers, direct genetic intervention through recombinant DNA methods will be used for improved synthesis and structure of polymers. Workers at the Massachusetts Institute of Technology have already taken a step in this direction. The ***Z. ramigera*** have been isolated that synthesize a new exopolysaccharide and have the ability to take up foreign DNA with conventional transformation techniques (Easson, Peoples and Sinskey, 1990). These mutant strains of ***Z. ramigera*** provide two useful tools for genetic enhancement of metal binding exopolysaccharide structure. First, the polysaccharide secreted by these mutant displays a 30 to 50% increase in the pyruvate content of the molecule. Since this mutation has a direct impact on the synthesis of a pyruvate rich polysaccharide, which should have increased metal binding properties, identification of these genes is simplified and mutation itself is useful. Secondly, this organism provides a genetic tool because, unlike the parent, it can take up DNA after simple and well-characterized treatments. The use of this new strain as a host for polysaccharide biosynthetic genes will permit study of gene expression in both ***Z. ramigera*** and other exopolysaccharide synthesizing microorganisms and has potential for producing new polymers by introducing novel genetic combinations into this organism.

2.4.4 Properties of Microbial EPS Relevant to Metal Binding

Many exopolysaccharides of aquatic microorganisms act as polyanions under natural conditions. Exopolysaccharides of most bacteria were examined, and found that they contain 5 to 25% uronic acids (Sutherland, 1980). Some sugar subunits of EPS poss ketal-linked pyruvate groups which generally contribute around 5 percent of the

polymer weight. Several of bacteria posses EPS containing sialic acids, which are derivatives of neuraminic acid. These also contain free carboxyl groups which confer net negative charge to the polymer (Geesey and Jang, 1989).

Polysaccharides also contain an abundance of hydroxyl groups which tend to interact with metal ions. The electronegative oxygen atom of hydroxyl group are likely to participate in metal interaction with anionic or neutral polysaccharides.

Some polysaccharides contain amino sugars or sugars with amide-link function group. These nitrogen-containing functional group are capable of reacting with some metals. However, the metal binding capacity contributed by the protein component of exopolymeric material involves these functional groups. Kihn et. al. (1987) showed that Cu^{2+} was chelated by peptides and protein extracted from wall of ***S. cerevisiae***. The binding site were formed by an amide and other strongly complexing, amine-like ligand. In slightly acidic conditions, Cu^{2+} was bound by oxygen of the amide, whereas at basic pH, $(NH_4)_2CO_3$ became deprotonated and the negatively charged nitrogen bound the metal (Geesey and Jang, 1989).

Metal adsorption by ***Z. ramigera*** is attributed mainly to its EPS, which composed of D-glucose, D-galactose and pyruvic acid in an approximately molar ratio of 11:3:1.5 should allow abundant binding site due to its slight negative charge and it many hydroxyl groups (Ikeda et. al., 1982).

2.4.5 Mechanisms of Metal Binding to Bacterial Exopolysaccharide

General interaction between a metal ion and an organic molecule may described as an acid-base reaction:

$$M^{n+} + LH \quad \leftrightarrow \quad M^{n+}\text{-}\ L + H^{+}$$

where M^{n+}, the metal ion, or H^{+}, the proton, represent the acid, and L, the organic molecule or ligand, represent the base. The release of proton by acidic polysaccharide during exposure to Cu ions has been reported in several instances (Zunino and Martin, 1977; Mittelmen and Geesey,1985). Binding of Cu ions resulted in a shift in the pKa of a capsular polysaccharide from 4.90 to 4.05 (Mittelman and Geesey, 1985). These data suggest that competition exists between Cu ions and protons at the metal-binding site of acidic exopolymers.

Metal ions tend to bind with functional group containing electron-donating atoms. Four type of interaction between metal ions and exopolymers have been described (Steiner, McLaren and Forster, 1976). The two most important type were those that involved salt bridges with carboxyl groups on acidic polymers and weak electrostatic bound with hydroxyl group on neutral polymer. In polysaccharide containing uronic acids or pyruvate groups, lone-pair electrons on oxygen atoms of carboxyl group have a strong tendency to interact with charge-compensating metal ions. Oxygen atom in the ether bonds and hydroxyl group of sugar subunits act as weak electron donor in both acidic and neutral polysaccharide (Martell, 1982). The "S-type" isotherm produced by complexes between metal ions and the capsule of ***Z. ramigera*** **115**

was proposed to involve primarily hydroxyl group of the glucose subunits and free carboxyl group on the ketal-link pyruvate residues (Brown and Lester, 1982). This type of metal binding is believed to be the most important mechanism of metal removal in activated sludge.

Manzini et. al. (1984) emphasized the importance of carboxyl groups in cupric ion binding. They suggested that interaction between Cu ions and carboxyl groups on acid polysaccharide. Cupric ion binding to a carboxyl group is electrovalent. In the case of polyuronates, the mode and extent of the interaction are believed to depend on several factors : the nature of the component sugar and their relative distribution in the chain, the magnitude of the over all electrostatic field, and the ratios of Cu to polymers and of Cu to simple supporting electrolyte. Under optimum condition 85 percent of the COO^- group can interact with Cu^{2+}. The remain 15 percent are inaccessible for complex formation. This is due to some extent to competition between Cu ions and couterions such as Na^+ for charged site on the polymer molecule.

Tan and Loutit (1976) investigated molybdenum binding by extracellular slime of rhizosphere bacteria. Greater than 90 percent of the Mo associated with cells of a ***Pseudomonas*** **sp**. was bound to the slime. Mo was mainly bound to the uronic acid of the slime layer. The slime from ***P. aeruginosa*** was reported to bind Mo through the glucuronic acid subunit (Stojkovski, Magee and Leisegang, 1986). Cu was also bound by this subunit. Glucuronic acid contributed 32 percent of the total slime weight. Complexion occurred through the two oxygen atoms of the carboxyl group and oxygen atom of the hydroxyl group of C-2 and C-4 of the uronic-acid molecule.

The floc polysaccharide extracted from ***Zoogloea* sp**. bound metal ions. Approximately 25 percent of the floc weight was contributed by bound metal ions. The polysaccharide produced by cell of ***Z. ramigera* 115** was reported to reduced the dissolved Cu to less than 0.1g/L. The complex metals could be released from the floc at pH 3-4. These results suggest that the metal were complex by carboxyl group on the polymers via ionic bonding (Geesey and Jang, 1990).

Metal uptake was compared between capsule-producing and noncapsulated strain of ***K. aerogenes***. Of the metals tested (Cu, Cd, Ni, Mn and Co), all but Ni were accumulated to a greater extent by the encapsulated strain. The extent of metal removal from solution by the capsulated strain followed the order Cu > Cd > Co > Mn > Ni. This sequence of affinity is similar to that displayed by ***Z. ramigera* 115** floc and to laboratory–scale activated sludge biomass (Rudd, Sterritt and Lester, 1983).

In summary, oxygen atoms of carboxyl and hydroxyl groups on the uronic acid subunits participate in the binding of metal ions by acidic capsular and slime polysaccharide. Oxygen atoms of hydroxyl groups on neutral sugar also contribute to the coordinate binding of metal ions that promotes the formation of stable complexes (Geesey and Jang, 1989).

2.4.6 Application of Bacterial Exopolysaccharide

Many industries and metal plating factories produce wastewater contain dissolved heavy metals such as Cu, Cd, Ni, Zn and Cr. Biomass and biopolymers have been used to complex the dissolved metals ions to a solid phase, detoxification or for recovery of valuable metal from

discharge water. For example, the AMT – BioClaim process, marketed by Advanced Mineral Technologies, Inc., Golden, Colorado, and represent successful to used biosorption in a technologically feasible way (Drierley, Drierley and Davidson, 1989).

Reactor design plays an importance role in determining the efficiency of metal recovery. The nature of the solid support will generally determine many of the reactor parameters. Since bacterial polymers can exist as gels, colloids and dissolved, they exhibit flexibility. The ability of bacterial exopolymers to exist in these different phases should maximize exposure of the binding sites to the metals dissolved in the bulk liquid phase. The flexibility of colloid and gel states should also provide advantages over other solid supports for ease of removal from the reactor (Geesey and Jang, 1989 and 1990).

One of the primary considerations of the solid support is its specificity or selectivity to certain metals. A great need to identify biopolymers that demonstrate high selectivity to metals, such as, Cu, Cd, Ni, Zn, Cr and Ag is presented. The minimal reactivity with cation such as Na, Ca, K and Mg is found. Manipulation of the culture condition and the genetic engineering of exopolymer-producing microorganisms should provide a useful approach to achieve this goal. In the future, microbial exopolymer should soon be identified with specific genes and their products so that the desired conformations can be selected. Other parameters such as binding–site density or binding capacity could be modulated at the gene level as well. Otherwise, there is an increasing demand for water used in processes in the electronics industry to contain less than 1 ppb of certain metal. "Polishing" industrial water and

effluents is still a major challenge to industry. One parameter that appears to be interesting in the case of bacterial exopolymers is the possibility of creating high diffusivity of metal ions within the polymer phase by manipulating the charge properties of the various functional groups on the polymer.

Although our current knowledge of interactions between bacterial exopolymers and metal ions is limited, the available information suggests that the polysaccharide have potential to form with metals that are important ecologically and economically. Research will help to advance our understanding of the role that bacterial polymers have in controlling the distribution of metal ions in nature and developed new technologies to use.

2.5 APPLICATION OF EXOPOLYSACCHARIDE

2.5.1 Living Cell Systems

Living cell system can be used to removal metal even though toxicity of metal is may be problem to used microorganisms, because of metal-resistant strain can reduce, accumulation and/or conversion metal to volatile and/or low toxicity by oxidation, reduction or methylation (Gadd, 1992). Otherwise, some mechanism are found in living cell, for example, volatilization, intracellular accumulation and extracellular precipitation.

Generally, living cell systems have been used for metal removal from wastewater contained metal at concentration below toxic level and may employ a mixture of microorganisms as well as higher plants

(Kauffman, Laughlin and Baldwin, 1986; Brierley, Brierley and Davidson, 1989).

2.5.2 Immobilized Systems

In commercial use, native biomass is low in mechanical strength, low density and small particle size, when native biomass is used in continuous stirred reactors its maximum clogging and after metal loading is difficult to separated biomass from solution, biomass must be separated by filtration, sedimentation or centrifugation, this is neither cost-effective nor efficient. So, must be improve characteristic. Immobilization serves to improve the characteristic of native biomass for suitable and economically used. After that, the biomass minimum clogging, easy to separated biomass from solution. Otherwise, its has particle strength, high porosity, hydrophilicity, resist to chemical and easy regeneration (Gadd, 1992).

Derived products, living cell or non-living cell can be used in immobilized form, microorganism can be immobilized in many supports such as agar, cellulose, silica, alginate, polyacrylamide, toluene diisocyanate, collagen, liquid membranes, metal hydroxide precipitates and glutaraldehyde.

2.5.2.1 Immobilized Bacterial Systems

Bacterial groups have been successfully in immobilized forms, for example, ***Bacillus* sp.** biomass has been used to removal heavy metal, the biomass is not selective and can remove many heavy metal, e.g., Cd, Cr, Cu, Hg, Ni, Pb, U and Zn. Single or mixed metal are generally loaded to > 10% of the dry weight, it has a metal removal efficiency

> 99% and effluents with total metal concentrations around 10 -50 ppb (Brierley, Brierley and Davidson, 1989).

Furthermore, immobilized living bacterial have been used as a biofilm on inert support, for example, ***P. aeruginosa*** was immobilized on particle of polyvinylchloride (PVC) and polypropylene webs and used in batch and column reactor for removal of heavy metals from wastewater (Hollo, Toth, Tengerdy and Johnson, 1979). Otherwise, another system used a mixed bacterial culture, mainly ***Pseudomonas* sp.**, immobilized as a film on anthracite particle for U removal.

2.5.2.2 Immobilized Fungal Systems

Fungal biomass has been used by immobilized with high-molecular-weight compounds, such as, gelatin, casein and other polypeptide materials. For example, ***A. oryzae*** has been immobilized on rectionlated foam. The immobilized fungal biomass was employed in a column contractor, it can removal 90% Cd as $CdSO_4$ in five min after metal solution contact with the biomass. The efficiency of removal is depend on pH, within the pH range of 5-8, it was found that the efficiency increase (Kiff and Little, 1986).

2.5.2.3 Immobilized Algal Systems

Algal biomass has been used by immobilizing with alginate and acrylamide. For example, ***C. vulgaris*** and ***S. platensis*** has been used to removal of metals, including Cu^{2+}, Pb^{2+}, Zn^{2+} and Au^{3+} from wastewater (Darnall et. al., 1986). Alginate and acrylamide have good resistance to hydrostatic pressure and mechanical degradation (Nakajima et. al., 1982), although acrylamide is non economically (Bedell and Darnall, 1990).

Otherwise, silica gel has been used to immobilized algal because of it has advantage in physical strength and cost. The immobilized algal product was very hard, resisted fragmentation and very porous (Darnall, 1988). In commercial, Alga SORB™ (Bio-Recovery Systems, Inc., Las Cruces, New Mexico 8803, USA) is preparation by immobilized algal biomass with silica matrix, it successfully to removal of Ag^{+}, Al^{3+}, Au^{3+}, Co^{2+}, Cu^{2+}, Cr^{3+}, Cr^{6+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Pt^{2+}, U^{6+} and Zn^{2+} from wastewater (Bedell and Darnall, 1990).

2.5.3 Metal Recovery

In commercial values, recovery and regeneration of biomass for used in multiple biosorption-desorption and capacity of biomass after recovery is very importance. The suitable method used for recovery from biomass depend on mechanism of accumulation. Metabolism independent biosorption is frequently recovery by non-destructive method, metabolism dependent, such as intracellular accumulation or binding to protein, etc. is often used destructive method by incineration or dissolution in strong acid or alkalis (Gadd, 1992). But in commercial values may used non-destructive method due to non destructive biomass, is result in maximum benefit in economically. For example, dilute mineral acid (0.1M) such as HNO_3, HCl, H_2SO_4 can removal of heavy metals from biomass, although at higher concentration (> 1M) or with prolonged exposure, it may be damage to biomass (Tsezos, 1984; Tsezos, Baird and Shemit, 1987). Other organic agents can used for desorption include EDTA, 8-hydroxyquinoline for copper and diethylenetriamine pentaacetic acid (DTPA) and nitriletriacetic acid (NTA) for cadmium (Gadd, 1992). Carbonate and/or bicarbonates are efficient desorption agents with potential for cheap, non-destructive although at high alkaline

pH of sodium carbonate solution can impair biomass structure (Tsezos, 1984).

Immobilized cell of ***Streptomyces albus*** with polyacrylamine can remove U, Cu and Co. In desorption, 0.1M Na_2CO_3 can used and not affected to biomass in five desorption cycles (Nakajima and Sakagushi, 1986). In U removal can used ***R. arrhiras*** biomass and desorption U from biomass by sodium bicarbonate ($NaHCO_3$); (Tsezos, 1984; Tsezos, McCready and Bell, 1989).

2.5.4 Industrial and Economic Aspects

In industrial purposes, biosorption may be used in a variety conditions, such as, batch, semi-continuous or continuous flow reactor and biomass are base on packed or fix-bed reactor systems.

Industrial application of biosorption depends on certain factors, such as, loading capacities, efficiency and selectivity and equivalence at least to physical and chemical treatment in performance (Tobin, Cooper and Neufeld, 1987, 1988; Tsezos, 1990; Brierley, 1989, 1990). Many biosorpbent appear competitive in efficiency and cost in physical and chemical treatment, e.g., ion exchange resin. Biosorpbent has high efficiency, operation over broad pH and temperature ranges (dead biomass or derived products) and multiple regeneration cycles (Voleskey, 1990). But, physical and chemical methods may be disadvantage include difficulties with suspended solid and land cost for evaporation, the requirement for low metal concentration, expensive and have a short working life for membrane process, high cost of chemical and difficult in disposal of contaminated sludge for precipitation technique, loss

adsorbent efficiency with prolonged use and regeneration for activated carbon, interference by Ca^{2+} and Mg^{2+}, chemical degradation and fouling, cost and sensitivity to thermal and osmotic shock and expensive for ion exchange resin (Voleskey, 1990), the suitable for high metal concentration in wastewater, expensive and have a short time working life of membrane for reverse osmosis process. Otherwise, biosorption has low cost, for example, fungi and yeast may cost \$1-5 kg dry $weight^{-1}$ and algae may cost \$ 15-18 kg dry $weight^{-1}$ but in ion exchange resin may cost \$15 - 31 kg dry $weight^{-1}$(Kuyucak,1990).