Removal of Copper Ion (Cu^{2+}) from Industrial Effluent by Immobilized Microbial Cells

by

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Abstract

A copper(II) accumulating *Pseudomonas putida* strain 5-X isolated from a heavy-metal-polluted area was studied for its potential use in copper removal from electroplating effluent. The copper removal capacity of the bacterium was enhanced by manipulation of culture conditions and physico-chemical pretreatments. The bacterium was then characterized for copper sorption properties. A laboratory scale bioreactor based on the immobilized cells of *P. putida* 5-X was installed to investigate the possibility of its use as biosorbent for copper removal .

The bacterium grown in a sulfate-limiting medium was found to result a removal capacity as high as 60 - 70 mg Cu /mg cell and bacterial cells cultured in this medium at 37 °C for 36 to 60 hours gave a high and stably maintained removal capacity. The copper uptake mechanism was metabolism-independent and the metal uptaken was largely located at the cell surface. Pretreatment by heat, acid and alkali did not enhance but decrease removal capacity of the cells under some conditions. Copper removal by *P. putida* 5-X was pH-dependent and removal capacity increased according to the rise in pH. Equi-molar concentration of nickel(II) ions did not inhibit copper(II) uptake while zinc(II) ions inhibited it slightly. The presence of lead(II) ions reduced copper(II) uptake by five-fold. Anions like borate, chloride and sulfate slightly inhibited copper(II) uptake while carbonate did not.

Cells of *Pseudomonas putida* 5-X was immobilized in polyacrylamide gel and packed in a column to serve as a reactor for copper removal. Retention time of copper-laden inffluent had little effect on the removal capacity of the immobilized cells. Copper loaded onto the immobilized cells could be recovered by 0.1 N HCl and 0.1 M EDTA solution by about 90 %. The immobilized cells could be used for 5 loading-elution cycles without loss of removal capacity. The ability to treat an electroplating effluent sample has been demonstrated.

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1. Objectives of the Study

Human activities involving heavy metals pose pollution problems to the environment and the situation is getting more and more serious. In Hong Kong, the major source of heavy metals is metal-finishing industries which release large amount of heavy metals including aluminium, chromium, copper, nickel and zinc. Removal of these metals from the industrial effluents at source before being released into the environment is necessary. In the present study removal of copper(II) ions from industrial effluents was targeted.

As the physico-chemical methods conventionally used for heavy metal removal are not satisfactory either economically or technically, alternatives or adjuncts to these methods are desired. Some microorganisms have been demonstrated to possess extraordinary capacity to accumulate heavy metals and the use of them in heavy metal removal is anticipated. In the present investigation, a bacterium *Pseudomonas putida* isolated from a heavy-metal-contaminated area was found to accumulate large amount of Cu(II) ions from aqueous solution. The copper removal capacity of the bacterium was enhanced by the manipulation of culture conditions and application of cell pretreatments. The bacterium was then characterized for its Cu(II) ion sorption properties. Finally a laboratory scale bioreactor model based on immobilized cells of the bacterium was evaluated for the feasibility of its use as biosorbent in Cu(II) ion removal from electroplating effluent. Possibility of subsequent copper(II) recovery and biosorbent regeneration was also investigated.

2. Literature Review

2.1. Heavy Metals in the Environment

Heavy metals are generally defined as metals with density greater than 5 g cm^{-3}. This rather arbitrary definition encompasses 69 elements of which 16 are synthetic and these metals are actually of very diverse chemical properties. Those which commonly cause pollution include antimony, arsenic, cadmium, chromium, cobalt, copper, iron, lead, mercury, nickel, silver, thallium, tin, vanadium and zinc (Martin and Coughtrey, 1982). As the term "heavy metals" usually implies toxicity, some metals which have a density lower than 5 g cm^{-3} are generally regarded as a member of the group, e.g. aluminium.

Toxicity of heavy metals to living organisms is well documented (Tyler *et al.*, 1989). Although some heavy metals are essential to life, e.g. chromium, copper, iron, manganese, molybdenum, nickel and zinc, they are required only by trace amounts and become toxic to living organisms when in excess. Moreover, although biomagnification of heavy metals is not evident, these metals are still transported along food chains and could enter human body (Patrick and Loutit, 1976, 1978; Förstner and Wittmann, 1981; Timmermans *et al.*, 1989). Besides posing threat to human health, toxic actions of heavy metals to other living organisms also causes perturbation of ecological processes in the biogeochemical cycles (Tyler *et al.*, 1989). As the heavy metals cycle through the environment while the environment provides no natural assimilative capacity for elements like metals, elevated heavy metal level in the environment poses risk to human health and the environment (Patterson, 1987).

Heavy metals are transported to air, water and soil from various sources through diverse routes. Natural sources release heavy metals from geochemical materials through various geological processes. Sources resulted from human activities include mining, agricultural activities, industrial processes and

miscellaneous activities in urban life. The metals are transported to the environment as mine wastes, pesticides, fertilizers, preservatives, sewage sludge and various forms of wastes produced by both metalliferous and non-metalliferous industries (Martin and Coughtrey, 1982). The metals released may be air-borne, contained in water runoff or flow, or dumped as solid residues (Patterson, 1987; Beijer and Jernelöv, 1986).

2.2 Heavy Metal Pollution in Hong Kong

Now in Hong Kong, 15 % of the total chemical waste produced annually was metal-containing wastes mostly as wastewaters generated by electroplating and metal-finishing industries (Environmental Protection Department, 1989). The composition of effluents from electroplating factories have been investigated (Chiu *et al.*, 1987). As shown in Table 1, aluminium, chromium, copper, nickel and zinc could occur at high concentrations. Moreover, the production of metal-containing wastes is expected to increase in the future. Table 2 presents the current and likely future metal-containing wastes generated in Hong Kong as assessed by the Environmental Protection Department (Environmental Protection Department, 1989). Among them copper-containing wastes are currently produced in particularly large amount and its production will also increase in the future.

However, in Hong Kong the importantce of treatment of metal-containing wastewates before their discharge has been ignored for a long time. These wastewaters are either channeled to the conventional wastewater treatment plant without pretreatment or just discharged directly to the environment and thus cause pollution (Reed, 1989). The problem of heavy metal pollution can be seen by the elevated level of heavy metals in Hong Kong coastal waters as revealed by the increasing amount of metals inside the shellfishes used as pollution indicators (Chan, 1989; Phillips, 1989). Measures would be required to remove the metals from wastewaters before their discharge.

Table 1. Metal content and pH of plating wastewater discharged from sixteen local electroplating shops (Adapted from Chiu *et al.*, 1987)

Parameters	Range
Chromium	1-40 mg/L
Copper	1-30 mg/L
Nickel	3-365 mg/L
Zinc	4-250 mg/L
Aluminium	10-230 mg/L
Silver	2-3 mg/L
pH	1.7-8.2

Table 2. Current and likely future metal-containing wastes arising in Hong Kong (Extracted data; Environmental Protection Department, 1989)

Metal-containing wastes	1988	1992 (tonne per annum)	1997
Copper-containing[1]	13,940	19,150	25,160
Zinc-containing[1]	3	13	14
Nickel-containing[1]	125	140	160
Chromium-containing[2]	55	59	68
Other Metals[1]	1,200	1,300	1,400

[1]As waste solutions

[2]As oxidizing agents

Besides toxicity of metal, the gradual depletion of metal resources also calls for the removal and subsequent recovery of heavy metals from metal-laden wastes. Some metals are expected to deplete in reserve and even get exhausted before 2100 (Goeller and Zucker, 1984). According to a system which ranks metals for priority of recovery with respect to their environmental risks and depletion rates (Table 3), zinc is ranked in high priority of recovery while chromium, copper and nickel are at medium position.

As in Hong Kong copper-laden wastewater is produced in the largest amount (see Table 2) and the recovery of this metal is desirable, in the present study removal and recovery of copper from electroplating effluents will be the metal targeted.

2.3. Chemistry and Toxicity of Copper in the Environment

2.3.1 General Chemistry of Copper

Copper is a transition metal with 3 common oxidation states: Cu^0 (metal), Cu^+ (cuprous ion) and Cu^{2+} (cupric ion). Cupric ion is the most commonly occurring species which readily forms free hydrated ion in water. It prevails in the environment and is the most toxic form to living organisms among the three. Cupric ion starts to precipitate above pH 5.5. But in the presence of organic or inorganic ligands, the ion get complexed and remains free in solution. It can be strongly complexed by electron donor groups (O-, N- and S-containing groups) in organic compounds (Flemming and Trevors, 1989).

Table 3. Example of prioritization for recovery of ten heavy metals (Adapted from Patterson, 1987)

Relative Priority	Environmental Risk*	Reserve Depletion Rate	Combined Factors
High	Cadmium Lead Mercury	Cadmium Lead Mercury Zinc	Cadmium Lead Mercury Zinc
Medium	Chromium Cobalt Copper Nickel Zinc	Aluminium Cobalt Copper Nickel	Cobalt Copper Nickel
Low	Aluminium Iron	Chromium Iron	Aluminium Chromium Iron

*Based on approximate relative toxicity

2.3.2 Toxicity of Copper

Like other heavy metals, toxic actions of copper on living systems are essentially exerted on enzymes, especially enzymes whose activities depend on sulfhydryl and amino groups, as heavy metals have high affinity for ligands containing nitrogen and sulfur donors (Morgan, 1987; Flemming and Trevors, 1989). Enzymatic activities may be inhibited by the binding of heavy metal ions to enzyme molecules, thus masking the catalytic sites, altering the conformations of the biomolecules, or competing with and displacing the enzyme-activating metal ions. Besides, nucleic acids and metabolites may also be targets of heavy metal toxic actions (Tyler *et al.*, 1989).

2.3.2.1 Toxicity of Copper on Human

The toxic effects of copper on human has been comprehensively reviewed by Aaseth and Norseth (1986). No chronic copper poisoning has been described in human beings. Acute copper poisoning after ingestion may show systemic effects like hemolysis, liver and kidney damage and fever with influenza syndrome. Local effects reported include irritation of upper respiratory tract, gastrointestinal disturbance with vomiting, epigastric burns and diarrhea, and a form of contact dermatitis. No evidence for correlation between copper exposure and cancer has been shown. Yet *in vitro* study found that cupric salts augmented the frequency of error during DNA replication. Copper can penetrate across the placental barrier. Copper-induced mal-development of central nervous system of human fetus has been reported.

2.3.2.2 Toxicity of Copper on Other Living Organisms in the Ecosystem

Impact of copper together with other heavy metals to ecological processes and individual groups of organisms in the environment are well-documented (Bååth, 1989; Flemming and Trevors, 1989; Påhlsson, 1989; Tyler *et al.*, 1989). In

terrestrial environment, the most easily observed toxic effect of copper is litter accumulation. Carbon mineralization and nitrogen transformation are both hindered. These effects are primarily due to toxicity of copper to soil microorganisms. Microorganisms toxified by copper may also reduce in abundance and biomass, shift in species composition and decrease in diversity (Bååth, 1989). Generally the order of toxicity of copper and other common heavy metals to microorganisms is cadmium > copper > zinc > lead. For soil invertebrates, commonly observable toxic effect of heavy metals are decreased species diversity. Reduced biomass or abundance, shifted species composition and changed vertical distribution along the soil profile are also found. The soft-bodied animals (e.g. earthworms and nematodes) and certain arthropod groups (e.g. oribatid mites) appear to be more susceptible to copper and other heavy metals (Tyler *et al.*, 1989).

Effect of copper to vascular plant has been extensively studied. Common physiological disturbances reported are accumulation of carbohydrates in foliages, stunted growth and chlorosis. Toxicity of copper along with other metals appears to decrease in the order cadmium > copper > lead ≥ zinc (Påhlsson, 1989). Susceptibility of bryophytes and lichens to copper varies largely. Field tests showed a sudden or gradual decrease in species abundance, diversity on exposure to copper and other metals. Their tissues are powerful ion-exchangers so that large amount of heavy metals can be accumulated (Tyler *et al.*, 1989).

Aquatic biota is especially susceptible to copper. Toxicological tolerance limits in fishes or crustaceans are generally 10 - 100 times lower than those in mammals and those in algae are even 1000 times lower. Copper inhibits photosynthesis of aquatic plants. Nitrogen fixation (mainly by cyanophytes) in surface waters is strongly inhibited by copper (Flemming and Trevors, 1989).

2.4 Conventional and Alternative Methods for Heavy Metal Removal

2.4.1 Effectiveness of Conventional Sewage Treatment Process in Heavy Metal Removal

Conventional sewage treatment process is often accountable for heavy metal removal from sewage although it is not purposefully designed to do so. The treatment process is accomplished by primary treatment (screening and sedimentation) followed by secondary treatment (activated sludge process and anaerobic sludge digestion). In primary treatment, precipitated metals are either settled or adsorbed onto settleable solids while soluble metals removal are usually minimal. Removal efficiency of heavy metals by primary treatment varies widely from one sewage treatment plant to another and from one metal to another. Average value is about 50 % with the exceptionally low efficiency in nickel removal by 24 % (Lester, 1983). The activated sludge process also give wide range of heavy metals removal efficiencies. Apparently iron, copper, chromium and zinc are most efficiently removed while nickel and manganese are not effectively removed through secondary treatment (Lester, 1983). Particulate metals are more efficiently removed than the soluble ones (Goldstone *et al.*, 1990a, 1990b, 1990c). Possible mechanisms of the removal process have been suggested to be (i) physical trapping of precipitated metal in the sludge floc matrix; (ii) binding of soluble metal ions by the bacterial exopolymers; (iii) accumulation of soluble metals by the cell; and (iv) volatization of metal to the atmosphere (Brown and Lester, 1979).

However, heavy metals can inhibit the biological processes involved in sewage treatment, i.e. activated sludge and anaerobic sludge digestion. Manifestations of heavy metal toxicity to activated sludge are declined BOD and COD removal (McDermott *et al.*, 1965), deflocculation of sludge, decreased species diversity, changes in communities of microorganisms and inhibition of nitrification (Lester, 1983; Sujarittanonta and Sherrard, 1981). Deflocculation can

make the activated sludge unsettleable and therefore the heavy metals contained in the solid phase cannot be separated from the sewage (Neufeld, 1976). Cases in which severe shock-loading of heavy metals posed ruinous effects in activated sludge systems have been reported (Lester, 1983). The general toxicity ranking of heavy metals to activated sludge process is nickel > zinc > chromium(VI) > copper > chromium(III) (Jackson and Brown, 1970). The anaerobic sludge digestion process is even more susceptible to heavy metal toxicity (Sujarittanonta and Sherrard, 1981) and the most sensitive part is methanogenesis where the key enzyme involved, coenzyme-M, is readily inhibited by heavy metals (Oleszkiewicz and Sharma, 1990).

It can be concluded that the conventional sewage treatment process would not be a reliable and ultimate method for heavy metal removal although its present role is important. As discussed above, both primary treatment and activated sludge perform variably in metal removal. It is not well suited to safeguard against the passage of metals to receiving water bodies, especially water reuse is to be practiced (Lester, 1983). Moreover, unrestrained heavy metal influx will toxified the biological processes involved so that both BOD and metal removal will fail. Subsequent sludge digestion will also be hindered and options in the ultimate disposal of the metal-concentrated sludge will be further limited (Hickey *et al.*, 1989). Actually the disposal sites could represent long-term reservoirs for the release of metals to the environment (Förstner, 1987).

2.4.2 Pretreatment Methods for Removal and Recovery of Heavy Metals from Metal-laden Wastewater

Due to the limited reliability of conventional sewage treatment process on heavy metal removal, pretreatment of metal-laden wastewaters before being discharged into sewers is desirable. Pretreatment methods used can be categorized into three kinds: A) conventional methods, B) substitute methods, and C)

emerging methods (Cushnie, 1985; Patterson, 1987). All of them are physico-chemical methods and most of them can remove copper as well as other metals. The following discussion will concentrate on methods which can be used for copper removal.

2.4.2.1 Conventional Methods

Conventional methods are those which have been most widely used and established as the standard in wastewater treatment. The primary principle of these methods is precipitation as hydroxides. One differs from the others by incorporating various accessory installations which serve to aid the precipitation process.

Most commonly used chemicals in precipitation are calcium hydroxide and sodium hydroxide. The insoluble metal hydroxides are then separated from the aqueous phase to achieve metal removal. Generally, operation is simple and the cost is low. The major disadvantage of this method is the production of a large volume of metal-laden sludge which is difficult to dispose of. The effluent produced often still contains metals at the mg/L level and the presence of complexing agents (e.g. ammonia and citric acid) further limits the metal removal efficiency. Some amphoteric metal hydroxides are insoluble only within a specific pH range and they will become soluble again when pH is out-ranged. Moreover, metal recovery is seldom possible due to economic factors (Cushnie, 1985).

2.4.2.2 Substitute Methods

Substitute methods are those proven feasible and preferable to conventional methods in particular situations. Some of them can effectively recover metals for further usage. These methods include freeze crystallization, evaporation, reverse osmosis, electrodialysis, electrolysis, cementation, ion exchange, starch xanthate adsorption and precipitation by sulfide and sodium borohydride. These substitute

methods are able to circumvent some problems encountered in the conventional methods. However, their application is still limited by economic or/and technological reasons, including high operation cost, fouling of components, impractical at low metal concentration, uneconomical or non-selective metal recovery, etc. (Netzer and Beszedits, 1979; Cushnie, 1985; Patterson, 1987).

2.4.2.3 Emerging Methods

Patterson (1987) suggested that differential precipitation and selective adsorption were emerging methods for metals removal and recovery. Differential precipitation involves multi-step titration of a complex metal-laden wastewater to precipitate out selected metals at different titration points. The method has been used to remove copper from a complex wastewater before a precious metal recovery step. Selective adsorption of heavy metals from wastewater by mineral oxides, polymeric resins and biological materials have also attracted much attention recently. The following section will discuss the advantages of using biological materials for heavy metal removal.

2.4.3 Heavy Metal Removal by Biosorbents

Heavy metal removal by living organisms or biological materials derived from them (which are thus called "biosorbents") has recently interested many researchers. The biosorbents apparently have the following advantages over the conventional sorbents for metal removal:

A) Their heavy metal removal capacity is apparently higher than that of conventional methods (Tsezos and Volesky, 1981; Brierley *et al.*, 1986b; Kuyucak and Volesky, 1988) and metal uptake can be selective.

B) The biosorbent used may be more economical. The plant materials are in abundant supply from nature (Chen *et al.*, 1990; Jana, 1988; Krishnan *et al.*, 1988; Kuyucak and Volesky, 1988; Kuyucak and Volesky, 1989; Sen and Mondal, 1989; Wright and Weber, 1991) while bacterial and fungal biomass may be supplied as waste in industrial fermentation processes and biological wastewater treatment (Shumate II *et al.*, 1980; Tsezos and Volesky, 1981; Tsezos and Keller, 1983). It is also possible to propagate the microbial biomass in wastes from various industries.

C) Biosorption processes are more effective in heavy metal removal than the conventional devices when the metal concentration in water is low (below 100 mg/L) and the effluent must contain less than 1 mg/L heavy metals (Shumate II *et al.*, 1978; Tsezos and Keller, 1983; Ross, 1989).

In the present study, the use of microorganisms as biosorbents for copper(II) ion removal was investigated. The following review will focus on the removal of heavy metals by various microorganisms.

2.5 Heavy Metal Removal by Microorganisms

Many microorganisms were found to accumulate large amount of heavy metals from the environment and have been considered for their use in heavy metal removal. Microorganisms studied include fungi (Norris and Kelly, 1977; Shumate II *et al.*, 1978; Baldry and Dean, 1980; Strandberg *et al.*, 1981; Tsezos and Volesky, 1981; Galun *et al.*, 1983; Mowll and Gadd, 1983, 1984; Tobin *et al.*, 1984; Townsley and Ross, 1985; Nakajima and Sakaguchi, 1986; Ragab *et al.*, 1986; Townsley *et al.*, 1986; de Rome and Gadd, 1987; Huang *et al.*, 1988; Kuyucak and Volesky, 1988; Gadd and White, 1989; Huang *et al.*, 1990), microalgae (Fisher, 1985; Gadd, 1988; Xue *et al.*, 1988; Harris and Ramelow, 1990) and bacteria (Tornabene and Edwards, 1972; Baldry and Dean, 1980; Aiking *et al.*, 1982; Marques *et al.*, 1982; Coleman and Paran, 1983; DiSpirito *et*

al., 1983; Macaskie and Dean, 1984; Norberg and Persson, 1984; Friis and Myers-Keith, 1986; Morozzi *et al.*, 1986; Nakajima and Sakaguchi, 1986; Bordons and Jofre, 1987; Wong and Choi, 1988; Goddard and Bull, 1989; Kasan and Baecker, 1989; Mullen *et al.*, 1989; Faison *et al.*, 1990; Van Dyke *et al.*, 1990). The present review will focus on microorganisms: fungi, microalgae and bacteria.

The ability of microorganisms to accumulate metals has been observed for a long time (Gadd, 1988). Many attempts have been made to screen for microorganisms which possess high metal removal capacity and then investigate their potential use in metal removal and recovery (Norris and Kelly, 1979; Baldry and Dean, 1980; Coleman and Paran, 1983; Nakajima and Sakaguchi, 1986; Kasan and Baecker, 1989; Campbell and Martin, 1990; Wnorowski, 1991). After many investigations, a general picture of the metal uptake phenomenon can delineated.

Heavy metals can be accumulated by microorganisms extracellularly, at the cell surface and intracellularly; in an active/metabolism-dependent way or a passive/metabolism-independent way. Different mechanisms may operate at the same time or in sequence. A commonly observed phenomenon in metal uptake kinetics is a biphasic process: a rapid, reversible, metabolism-independent association of metal ions with the cell surface or exopolymers, followed by a slow, metabolism-dependent transport of metal ions across the cell membranes into the cytoplasm, where accumulated metals are not easily released (Brierley *et al.*, 1985; Gadd, 1988). Figure 1 depicts the generalized biphasic metal uptake process by microbial cells.

In the following review, metal uptake processes by microbes will be first classified into active/metabolism-dependent and passive/metabolism-independent mechanisms. Studies on the two mechanisms found in fungi, microalgae and bacteria will then be discussed, followed by cases of proposed or realized application for heavy metal removal wherever reported.

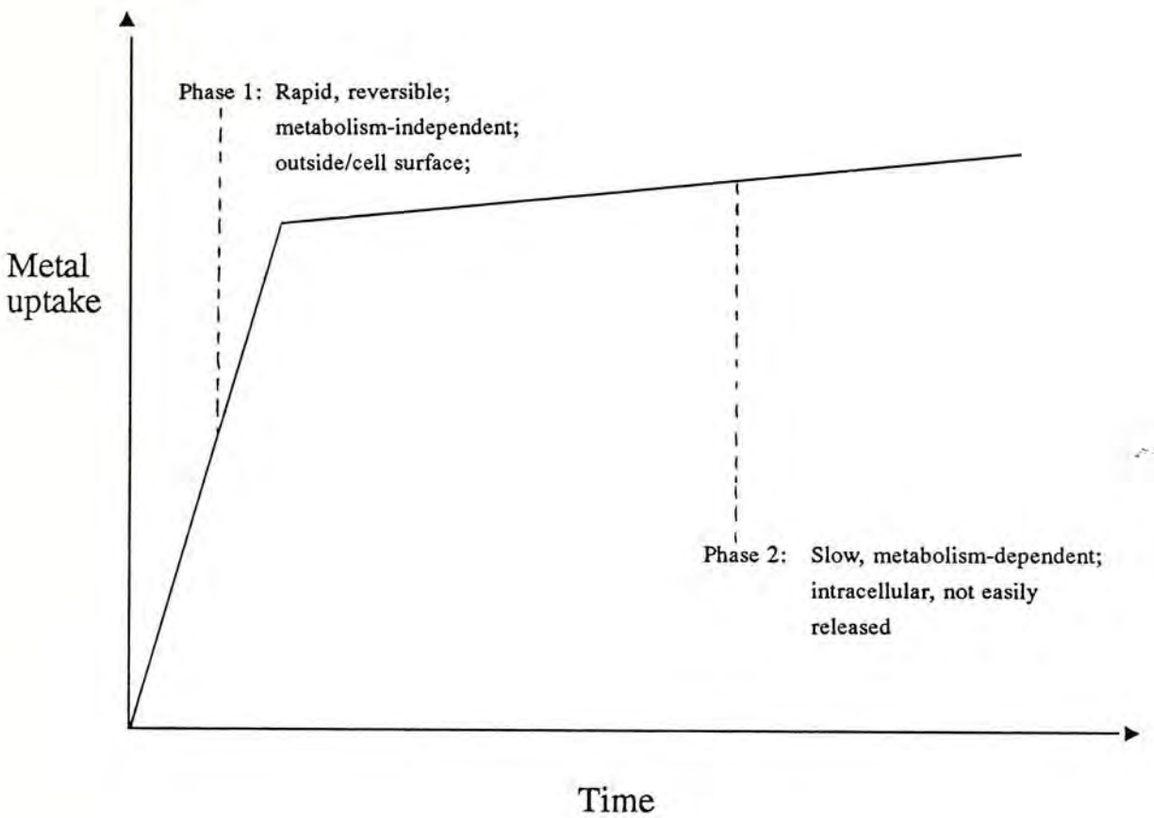

Figure 1. Generalized biphasic metal uptake process in microbial cells. Adapted from Brierley *et al.* (1985) and Gadd (1988).

2.5.1 Metabolism-dependent Metal Uptake by Microbial Cells —Bioaccumulation

The ability of microorganisms to accumulate metals in the natural environment has long been studied from a toxicological point of view, with focus on the metals' effects on the metabolic activities of the microbial cells and the significance of metal accumulation along the food chain. The uptake of metals by living microbial cells could be termed "bioaccumulation" (Volesky, 1987). Microbial cells can accumulate heavy metals in a metabolism-dependent way by mainly three mechanisms: metabolism-mediated precipitation and redox reactions, and transportation through ion transport systems.

2.5.1.1 Metabolism-mediated Precipitation of Heavy Metals

Many microorganisms are able to produce hydrogen sulfide which react with heavy metals to form insoluble metal sulfides. These include the sulfate-reducing bacteria (Hutchins *et al.*, 1986), *Klebsiella aerogenes* (Aiking *et al.*, 1982, 1984, 1985) the yeast *Saccharomyces cerevisiae* (Minney and Quirk, 1985), and the alga *Cyanidium caldarium* (Wood and Wang, 1985). The accumulated metal sulfides are either trapped intracellular or bound at the cell surface. In all cases, the precipitation of heavy metals can act as a detoxification mechanism and render the microorganisms metal resistant.

Metal sulfide formation effected by sulfate-reducing bacteria acts as important component in some "natural-setting" systems for heavy metals removal from water in some lake and streams (Brierley *et al.*, 1989). The anaerobic sulfate-reducing bacteria in the sediment produce hydrogen sulfide which precipitate the heavy metals (Brierley *et al.*, 1989; Hutchins *et al.*, 1986). Purposefully built system based on the above principle was also reported (Brierley *et al.* 1989).

Heavy metals may also be precipitated as phosphate. A cadmium resistant *Citrobacter* sp. synthesized a cell-bound phosphatase induced on growth in medium

with glycerol phosphate as sole phosphate source. Phosphate produced by the phosphatase precipitated cadmium on the cell surface as cadmium phosphate, rendering the bacterium cadmium resistant (Macaskie and Dean, 1984; Macaskie *et al.*, 1987a). The bacterium has been immobilized to remove heavy metals like cadmium, uranium, copper and lead (Macaskie and Dean, 1987; Macaskie *et al.*, 1987b; Ross, 1989). However, it seems that no industrial nor pilot scale of this system has been tested (Ross, 1989).

2.5.1.2 Metabolism-mediated Oxidation-reduction of Heavy Metals

Bacteria are able to oxidize or reduce inorganic compounds and accumulated them as insoluble compounds. Bacteria like *Gallionella* sp. and *Thiobacillus ferrooxidans* can oxidize Iron(II) to obtain energy and deposit oxidized iron compounds on their long twisted stalks. *Leptothris discophora*, *Lactobacillus plantarum*, species of *Pseudomonas*, *Citrobacter*, *Arthrobacter*, *Metallogenium*, phytoplanktons and fungi oxidize manganese compounds to manganese oxides and accumulate them at the cell surface through the actions of manganese oxidizing proteins or the production of oxidants (Nealson *et al.*, 1989). Deposition of manganese oxides by unknown mechanisms was also found in other microbes (Gadd, 1988).

Recently a laboratory-scale reactor using growing cells of *Gallionella ferruginea* and a *Sphaerotilus* sp. for iron removal has been described (Viswanathan and Boettcher, 1991). The iron-oxidizing bacteria grew and attached to a sand-gravel column where iron(II)-containing groundwater was passed through. The iron(III) resulted from microbial oxidation was allowed to be further hydrolyzed to insoluble $Fe(OH)_3$ in a detention tank and the effluent was filtered before discharge. The iron removal efficiency could reach 90% (effluent iron concentration = c.a. 0.3 mg/L) after 14 days of operation.

Chromate is reduced to insoluble chromium(III) compounds by some

bacteria, e.g. certain *Pseudomonas* spp. and a strain of *Enterobacter cloacae*. The reduction reaction may occur in aerobic or anaerobic condition (Wang *et al.*, 1989; Ishibashi *et al.*, 1990; Komori *et al.*, 1990b). It was proposed that the *Enterobacter cloacae* strain used chromate as the terminal electron acceptor in the respiratory chain under anaerobic condition (Wang *et al.*, 1989). Chromium(III) compounds formed are suspended in solution but whether they are also accumulated by the bacterial cells was not reported. Recently a laboratory-scale model of chromate removal device based on the reduction activity of *Enterobacter cloacae* has been preliminarily tested (Komori *et al.*, 1990a).

2.5.1.3 Transportation of Heavy Metals through Ion Transport System

Heavy metals may be accumulated intracellularly through active transport. Some heavy metals are essential to microbial metabolism and they can be transported into the cytoplasm via specific ion pumps, e.g. manganese, nickel and zinc. Others with no known physiological function, like cadmium and chromate, can enter the cytoplasm through transport systems specific for the essential ions. Cadmium (Cd^{2+}) may compete with manganese (Mn^{2+}) for their specific transport systems in Gram-positive bacteria and use the zinc transport system to enter the *Escherichia coli* cells. Chromate ion (CrO_4^{2-}) go into cells of *Pseudomonas fluorescens* through the sulfate-transport system (Ohtake *et al.*, 1987).

Many iron-dependent microorganisms produce extracellular iron binding molecules called siderophores. The complex formed by iron and siderophore interacts with the cells so that iron is accumulated intracellularly (Gadd, 1988). Siderophores has been prepared as commercial product after immobilization on substrate such as silicate. This product was reported to have high affinity to specific metals. It can be packed in column for use, stripped of the bound metals and regenerated for reuse some 100 times without deterioration (Brierley *et al.*, 1989).

Heavy metals entered cytoplasm may be detoxified by forming inorganic deposit or binding to specific proteins. *Klebsiella aerogenes* detoxified incoming cadmium by forming insoluble cadmium sulfide or cadmium phosphate deposits inside the cell (Aiking *et al.*, 1982, 1984, 1985). Similar phenomena are observed in cyanobacteria, eukaryotic algae, yeast, etc. (Wood and Wang, 1985; Gadd, 1988). Metal binding proteins found usually bind copper, zinc or cadmium specifically. Cases have been reported in bacteria, cyanobacteria, fungi and microalgae and some of these proteins are metallothionein-like (Olafson *et al.*, 1979; Higham and Sadler, 1984; Murasugi *et al.*, 1984; Butt and Ecker, 1987; Gadd, 1988; Sakamoto *et al.*, 1989). The possibility of utilizing metal binding proteins in heavy metal removal was suggested (Butt and Ecker, 1987) but no such application has yet been reported.

2.5.2 Metabolism-independent Metal Uptake by Microbial Cells —Biosorption

Cases of metabolism-independent uptake of heavy metals by microbial cells are well-documented and such kind of uptake process is often described as "biosorption". Despite the rather specific definition given by Shumate II and Strandberg (1985), biosorption of heavy metals is now generally referred to the non-directed physico-chemical interactions that occur between metal/radionuclide species dissolved in aqueous phase and cellular components of living organisms which act as a solid phase. Interactions involved may be physical adsorption, ion-exchange, complexation, precipitation, crystallization or diffusion (Shumate II and Strandberg, 1985; Gadd, 1988). The following review will focus on the biosorption mechanisms of microorganisms, with emphasis on particular cases which have been detailedly studied.

2.5.2.1 Biosorption in Bacteria

Bacterial cell walls are generally negatively charged and therefore their

ability to bind heavy metal cations is expected. Gram-positive bacteria possess a peptidoglycan framework whose peptide stems contain carboxyl groups, rendering the cell wall anionic. Techoic acid and techuronic acid, two other common components of bacterial cell wall, confer net negative charge to the wall by the phosphate groups they contain. Gram-negative bacteria have only a thin peptidoglycan layer sandwiched by a outer membrane and a plasma membrane. The outer membrane consists of a mosaic of phospholipids, lipopolysaccharides and proteins. Both phospholipids and lipopolysaccharides appear to be anionic (Beveridge, 1989).

Other superficial layers of bacteria can also contribute to metal ion binding. Capsules composed of polysaccharides or polypeptides usually give a net negative charge due to the presence of carboxylates or phosphate groups (Beveridge, 1989). The S-layers which contain acidic proteins play an important role in heavy metals accumulation and detoxification by *Sporosarcina ureae* (Beveridge, 1984). Sheaths, which are uncommon in bacteria, contribute much to iron/manganese oxides formation in the environment (Gadd, 1988; Beveridge, 1989).

2.5.2.1.1 Heavy Metal Uptake by Bacterial Cell Wall

The metal uptake mechanisms of cell wall of the Gram-positive *Bacillus subtilis* and the cell envelope of the Gram-negative *Escherichia coli* has been thoroughly studied. Cell wall of *B. subtilis* bind metals mainly by peptidoglycan, with techoic acid playing the minor role (Beveridge and Murray, 1980). But one should not over-generalize the observation as *B. licheniformes* differs from *B. subtilis* by having techoic acid and techuronic acid as major metal binding component instead of peptidoglycan (Beveridge *et al.*, 1982). *B. subtilis* was found to have its surface metal concentrations far exceeding the expected stoichiometry of metal bound per reactive chemical site within the cell wall and metal precipitates were visible under electron microscope. A two-step mechanism of metal uptake

was thus proposed: firstly there was a stoichiometric interaction of metal with reactive chemical groups which primarily exist in the peptidoglycan; then the bound metal ion act as a nucleation site for the deposition of more metal ion from solution to form a chemical precipitate (Beveridge, 1984).

E. coli K-12 has also been studied for its metal binding ability. Whole cell of the bacterium in natural environment binds metals mainly at the outer membrane surface but not the thin peptidoglycan layer. The prime candidate for metal ion binding sites in isolated cell wall is the phosphate groups intrinsic to the lipopolysaccharides and phospholipids. The isolated peptidoglycan sacculus of *E. coli* accumulates metals as efficient as the peptidoglycan wall in *B. subtilis* and it binds more metals than the outer membrane does (Beveridge, 1984).

An immobilized cells system using *Bacillus* species has been developed for heavy metal removal. Bacterial cells of *Bacillus* species used in fermentation for production of enzymes and other chemicals are alkali-treated and immobilized to form a non-living granules which bind heavy metals like cadmium, chromium, copper, lead, mercury, nickel, uranium and zinc but not the non-toxic metals (calcium, magnesium, potassium, sodium). The granules sorb the metals with a loading which exceed 10% of their dry weight, remove metals from solution with over 90% efficiency and produce an effluent with 10-50 μg/L total metal concentration (Brierley *et al.*, 1986a, 1987). A wastewater treatment system utilizing these granules has been developed. The granules can be used in a dispersed bed contactor and regenerated for use with the bound metals recovered (Brierley *et al.*, 1989).

2.5.2.1.2 Heavy Metal Uptake by Bacterial Exopolymers

Bacterial exopolymers are another cellular component studied most for their metal binding properties. The role of these exopolymers in metal removal by

activated sludge has been well-documented (Brown and Lester, 1979; Lester *et al.*, 1984; Lester, 1987). Several bacteria isolated from the activated sludge, such as the *Zoogloea* spp., *Pseudomonas* spp., *Klebsiella aerogenes* and *Aerobacter cloacae*, are found to produce these polymers. The polymers may be firmly attached to the cell wall to form a capsule surrounding the whole cell. Some polymers are slime-like and continually diffused to the aqueous phase from the cell surface (Bitton and Freihofer, 1978; Lester, 1987).

The chemical nature of exopolymers extracted from activated sludge and pure bacterial cultures has been studied. Since relatively large amount of polysaccharides or monosaccharides were produced on hydrolysis, these exopolymers are frequently termed exopolysaccharides. Most common monosaccharides found in exopolymers are D-glucose, D-galactose and D-mannose. Besides these uncharged components, charged components like uronic acids and hexosamines are also found (Brown and Lester, 1979). These charged components could have contributed to binding of heavy metal ions by exopolymers. A study on uranium removal by isolated exopolymers from a *Pseudomonas* sp. showed that the anionic carboxyl groups might be responsible for metal binding (Marqués *et al.*, 1990).

Exopolymers from *Zoogloea ramigera* has been extensively studied. *Z. ramigera* produces large amount of exopolymers in the form of capsule or "zoogloeal matrix". These exopolymers are responsible for the floc formation in activated sludge and able to accumulate large amount of heavy metals (Brown and Lester, 1979). The exopolymers produced by *Z. ramigera* 115 has been proposed to be a highly branched hetero-polysaccharide composed of glucose, galactose and pyruvate (Ikeda *et al.*, 1982) but another study disagreed with the proposal (Franzen and Norberg, 1984). Although the results on chemical structure of exopolymers are not consistent, the ionizable carboxyl groups of pyruvate are generally believed to be responsible for binding of heavy metal ions (Ikeda *et al.*, 1982; Franzen and

Norberg, 1984; Sinskey *et al.*, 1986).

The use of exopolymers in heavy metal removal has been anticipated. Norberg and Persson (1984) studied the removal of heavy metals by a *Zoogloea ramigera* culture which bound and separated heavy metal ions from the liquid phase by a low-pH-induced flocculation (pH = 5.5). In the batch type experiment, it was reported that copper, cadmium and uranium could be loaded onto the *Z. ramigera* biomass up to 40%, 50% and 80% of its dry weight respectively. A laboratory-scale continuous process based on the principles of the batch system has been developed to remove copper. The process was described to be less effective than the batch system (Norberg and Rydin, 1984). *Z. ramigera* has also been immobilized in calcium alginate beans and used in a bubble column to treat a solution of mixed metals. The overall metal removal efficiency of the system could attain over 95.9 % removal of the metals. The efficiency could be enhanced by treating the heavy metal containing water in sequentially linked reactors. However, the specific uptake capacities of these metals by the biomass has not been reported (Kuhn and Pfister, 1989).

2.5.2.2 Biosorption in Fungi

As fungal biomass is able to accumulate heavy metals and is readily obtainable from fermentation industries as waste materials, it is suggested that fungal biomass can be used as biosorbent for heavy metal removal from wastewater (Tsezos and Volesky, 1981; Tsezos and Keller, 1983; Luef *et al.*, 1991). Among these attempts, the biosorption of uranium and thorium by *Rhizopus arrhizus* has been thoroughly studied.

Tsezos and Volesky (1981) found that a strain of *Rhizopus arrhizus* accumulated uranium and thorium up to 0.18 g uranium and 0.17 g thorium per g dried cell and such uptake capacities were found to be higher than that of some activated carbon and ion-exchangers currently used for uranium removal. The

amount of metals sorbed far exceeded the expected stoichiometry of metal uptake per reactive chemical site within the cell wall. Under electron microscope metal-containing granules were found to associate with the fungal cell wall, as in the case of *Bacillus subtilis*. Chitin in the cell wall was suggested to be largely responsible for the interaction with the metals. A three step mechanism for uranium uptake was proposed (Tsezos and Volesky, 1982a; Tsezos, 1983):

(A) amino groups of the cell wall chitin network act initially as uranium coordination sites,

(B) the coordinated uranium serves as nucleation site for adsorption of additional uranium, and

(C) adsorbed uranium precipitates as uranyl hydroxide within the cell wall matrix.

A study conducted by Tobin *et al.* (1984) supported the suggestion that the initial step was complexation of uranyl ion, but another study indicated that uranyl ion associated with chitin mostly by ion-exchange (Treen-Sears *et al.*, 1984b).

On the other hand, biosorption of thorium may involve two processes which are not interactive as in uranium uptake (Tsezos and Volesky, 1982b):

(A) formation of coordination complex between thorium and the nitrogen of the cell wall chitin, and

(B) adsorption of hydrolyzed thorium ions by the outer layers of the cell wall, probably to reactive sites other than the complexation sites

A laboratory scale pilot plant based on immobilized biomass of a strain of *R. arrhizus* has been reported. The immobilized fungal biomass was used in a packed bed reactor and the activity of the biosorbent only dropped by 40% after 12 uranium loading-elution cycles. Recovery of uranium from the biosorbent was over 90% in most cases (Tsezos *et al.*, 1989).

The yeast *Saccharomyces cerevisiae* is another fungus which has attracted a lot of attention to its application in biosorption because it is highly availability from fermentation industries. In the studies using growing cells of *S. cerevisiae*, sequestration of cadmium, cobalt and zinc by the yeast cells were found to be biphasic: a rapid, metabolism independent binding of metal ions to the cell surface, followed by a progressive, metabolism-dependent intracellular uptake of metal ions via a general cation transport system (Norris and Kelly, 1977; Mowll and Gadd, 1983). Such biphasic process was also observed in immobilized resting cells of *S. cerevisiae* and uptake of strontium by the metabolism-dependent process was enhanced by the presence of glucose (de Rome and Gadd, 1991). However, an earlier study on uranium uptake by free resting cells showed that intracellular uranium uptake was very rapid and unaffected by metabolic inhibitors (Strandberg *et al.*, 1981). No study on metal biosorption mechanism in *S. cerevisiae*, which is as thorough as that conducted by Tsezos on *Rhizopus arrhizus*, has been accomplished. Laboratory-scale metal removal devices based on immobilized cells of the yeast have been described but these works remained preliminary (Huang *et al.*, 1990; de Rome and Gadd, 1991).

2.5.2.3 Biosorption by Microalgae

Investigations on bioaccumulation of heavy metals by microalgae are not uncommon but studies which aim at biosorption of heavy metals as a means of environmental clean up are much rarer (Gadd, 1988). The most popular microalgae studied for its biosorptive capacity is *Chlorella*. Its removal capacity for aluminium, cadmium, copper, gold, silver and zinc has been studied (Darnall *et al.*, 1986; Greene *et al.*, 1986; Harris and Ramelow, 1990). Mechanistic study on the metal biosorption by the alga are very limited. A preliminary experiment showed that sulfhydrl groups might be involved in the binding of silver (Darnall *et al.*, 1986). A few metal removal devices using microalgae has been developed but all

of them were only laboratory scale model studies (Darnall *et al.*, 1986; Harris and Ramelow, 1990).

2.5.3 Advantages of Biosorption over Bioaccumulation in Heavy Metal Removal

In the early attempt to use microorganisms for heavy metals removal, growing cells were extensively studied. Heavy metal resistant microorganisms were isolated and screened for their heavy metal removal capacity during their growth in the presence of heavy metals. Treatment of wastewaters containing both organic nutrients and heavy metals by growing cells of the metal accumulating microorganisms was envisaged as BOD and heavy metal levels could be reduced together on the growth of the microorganisms.

However, separation of the cell growth and accumulation process was proposed later (Shumate II *et al.*, 1978) because the decoupling of the two stages has several advantages. In a growing cell system, it takes time for microorganisms to grow up to the biomass concentration required for adequate heavy metal removal while growth in toxic metal ions may be even slower. Metal removal process using resting or dead cells will be much quicker because biosorption by pregrown biomass can complete the metal removal process within a very short period of time. On the other hand, heterogeneity of metal-laden wastewater changes the growth conditions of the microbial cells which in turn affect the metal removal capacity of the cells (as discussed in section 2.6.1). The growing cells, even though heavy metal resistant, may also be toxified by hazardous matters other heavy metals present in the wastewater. By using microbial cells pre-grown in optimal conditions, such problem can be circumvented (Shumate II *et al.*, 1978). The present investigation was thus focused on biosorption as a means of heavy metal removal.

2.6 Factors Affecting Biosorption of Heavy Metals

Numerous studies show that effectiveness of biosorption can be affected by

many factors, namely, cultural conditions of the microbial cells, pretreatment of the microbial cells before biosorption, and the environmental conditions of the biosorption process.

2.6.1 Effect of Culture Conditions

Since most biosorption processes involve the microbial cell surfaces while culture conditions of microbial cells like growth rate, medium composition, culture pH, oxygen and carbon dioxide concentrations, incubation temperature, etc. are found to influence cell surface properties (Hancock and Poxton, 1988), it is reasonable to expect that some of these conditions can affect the biosorptive properties of microbial cells.

The age of culture of microbial cells has been found to affect their biosorption properties. *Zoogloea ramigera* culture of six to eight days old removed cadmium and copper two times more than a two-day culture but its removal capacities dropped again after the eighth day of culture (Norberg and Persson, 1984). Maximum amount of cadmium was removed by an *Arthrobacter viscosus* culture which had been grown for 48 hours or more. This higher removal of cadmium probably due to the maximal synthesis of exopolysaccharides by the bacterium at that time (Scott and Palmer, 1990). *Rhizopus arrhizus* removed a decreasing quantity of uranium as the culture age of the fungus increased after 20 hours of incubation (Treen-Sears *et al.*, 1984b).

Medium composition can also influence biosorptive properties of microbial cells. An *Enterobacter* sp. grown in a phosphate-limiting medium removed almost twice as much nickel as the bacterium grown in glucose-, ammonia- and sulfate-limiting media did (Kwok, 1990). Carbon/nitrogen ratio of culture medium affected the cell surface composition of *Aspergillus oryzae* and an C/N ratio of 10 - 15 was found to be optimal for metal uptake by the fungal biomass propagated (Huang *et*

al., 1989). *Rhizopus oligosporus* sequestered less uranium when the growth medium had neopeptone totally substituted by peptone (Treen-Sears *et al.*, 1984b). Augmentation of mineral salts (including several trace elements) level in a growing culture of *Rhizopus javanicus* increased the biomass concentration and copper uptake by the biomass produced was enhanced (Treen-Sears *et al.* 1984a). Culture pH has also been reported to affect the copper removal capacity of the *R. javanicus*. As the fungal biomass was a effective biosorbent for metals, a decreased pH might dissociated the trace elements from the biomass and increased their availability to the fungus.

2.6.2 Effect of Pretreatment of Microbial Cells

Various physico-chemical pretreatments of microbial cells have been investigate for their effects on their subsequent biosorptive properties. Alkali treatment was found to enhance metal uptake by a *Bacillus* biomass (Brierley *et al.*, 1989), uranium uptake by *Penicillium digitatum* (Galun *et al.*, 1987) and strontium uptake by *Micrococcus luteus* (Faison *et al.*, 1990) but did not affect cadmium uptake by *Aspergillus oryzae* (Huang *et al.*, 1988). Heat treatments (around 100°C) increased strontium uptake by *M. luteus* and uranium uptake by *P. digitatum* (Galun *et al.*, 1987) but reduced copper uptake by *Saccharomyces cerevisiae* (Huang *et al.*, 1990). Acid treatment augmented copper uptake by *S. cerevisiae* (Huang *et al.*, 1990) but suppressed strontium uptake by *M. luteus* (Faison *et al.*, 1990). Ethanol treatment elevated the uranium removal capacity of *P. digitatum* (Galun *et al.*, 1987) and the copper removal capacity of *S. cerevisiae* (Huang *et al.*, 1990). Other uncommon treatments included surfactants treatments (Faison *et al.*, 1990), dimethylsulfoxide and formaldehyde treatments (Galun *et al.*, 1987). Explanations to the effects of various pretreatments were mostly hypothetical. Moreover, the contradictory effects of the apparently equivalent treatment procedures may not only be due to structural differences between different kinds of microorganisms but also variations in the details of pretreatment procedures.

2.6.3 Effect of Environmental Conditions

Biosorption involves certain physico-chemical interactions between the metals and the cellular components and therefore is influenced by environmental conditions which generally affect physico-chemical processes (Shumate II and Strandberg, 1985). Conditions whose effects are commonly investigated included pH, temperature, initial metal concentration, presence of competing cations, and presence of anions or ligands.

Generally, metal removal capacity of a biosorbent is reduced at low pH due to the presence of high concentration of competing hydrogen ions. But the removal capacity does not necessarily increase according to an elevating pH and optimal pHs for uptake of particular metals by specific biosorbents are usually found. Optimal pH for copper uptake by native cells of *Saccharomyces cerevisiae* was about 5.5 and copper uptake decreased when pH raised further (Huang *et al.*, 1990). Optimal pH for cadmium uptake by *Fusarium solani*, *Aspergillus pullulans* and *Aspergillus oryzae* were approximately 6, 7 and 8 respectively (Huang *et al.*, 1988). Raised temperature (within the range 0-100°C) enhanced biosorption by increasing metal uptake rate (Shumate II *et al.*, 1978; Kuhn and Pfister, 1990) and removal capacity at equilibrium (Tsezos and Volesky, 1981; Brierley *et al.*, 1986b). Inhibition of biosorption by temperature near ice point was evident in copper removal by *Trichoderma viride* (Townsley *et al.*, 1986) but no prominent effect was found on copper uptake by *Rhizopus arrhizus* (de Rome and Gadd, 1987).

Inhibition of biosorption of heavy metal by the presence of other metals is generally observed. Usually monovalent alkali metal ions (e.g. Li^+, Na^+, K^+) are weak inhibitors while the divalent and trivalent metal cations (e.g. Ca^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}; Al^{3+}, Fe^{3+}, etc.) are much stronger ones (Gadd and White, 1989; Faison *et al.*, 1990). A study indicated that the metal cations competed for binding sites on the biomass of *Rhizopus arrhizus*, but different metals might prefer

different binding sites (Tobin *et al.*, 1988). The presence of anions may also inhibit the uptake of metal ions. Ethylenediaminetetraacetic acid (EDTA), citrate and nitriloacetate extensively remobilized strontium bound to the cells of *Micrococcus luteus* but carbonate did not (Faison *et al.*, 1990). Aspartic acid and glutamic acid reduced uranium uptake rate and capacity of *Saccharomyces cerevisiae* but other monocarboxylic and sulfur-containing amino acids tested had no effect (Strandberg *et al.*, 1981). Tobin *et al.* (1987) reported that uranium uptake was only slightly inhibited by sulfate, chloride, phosphate, glutamate and carbonate but inhibition was strong when EDTA was present.

2.7 Applicability of Biosorbent in Heavy Metal Removal

For a biosorbent to treat heavy metal containing wastewater effectively, it should fulfill a number of criteria which are requisite as well to conventional sorbents (Volesky, 1987; Gadd, 1988; Ross, 1989):

(1) The biosorbent should possess high heavy metal removal capacity and metal uptake rate.

(2) The biosorbent should have appropriate size, shape and physical properties which allow efficient separation from the treated solution and can be used in a continuous flow system.

(3) The biosorbent should be produced at low cost and reusable without significant loss of activity.

(4) The metals sorbed by the biosorbent should be economically recoverable.

The fulfillment of high metal removal capacity of microbial biomass-derived biosorbent can be achieved by manipulations by culture conditions and cell pretreatment. The metal uptake rate of biosorbent is often as quick as conventional sorbents and such requirement is not difficult to achieve. For the other

requirements, they can be met directly or indirectly by using immobilized microbial cells as biosorbent.

2.7.1 The Use of Immobilized Cells as Biosorbent

The advantages of using immobilized microbial cells in bioreactor are well-documented (Smith, 1985). An advantage particularly relevant to biosorption of heavy metals is the efficient separation of the immobilized cells from the treated metal-laden wastewater. This also implies the convenient separation of metal containing eluant from the biosorbent after a metal recovery step. The material properties of a biosorbent may also be manipulated by proper choice of immobilization methods. For example, the immobilized cells of macroscopic size are more easily retained in a bioreactor operated in a continuous-flow mode (Volesky, 1987) and pipeline blockage and filter clogging by the freely suspending microscopic microbial cells can be avoided (de Rome and Gadd, 1991). The increased stiffness of immobilized cells will also be more abrasion resistant when used in a stirred tank or fluidized bed reactor (Volesky, 1987).

Microbial cells used for biosorption have been immobilized in a number of methods. Cells of *Saccharomyces cerevisiae* and mycelia of *Trichoderma viride* have been immobilized by embedment in sand and molochite respectively (Townsley *et al.*, 1986; Huang *et al.*, 1990). *Zoogloea ramigera* and *Chlorella vulgaris* were entrapped in calcium alginate and polyacrylamide respectively to remove various metals (Darnall *et al.*, 1986; Kuhn and Pfister, 1989). Cells of an *Citrobacter* sp. has been immobilized as a biofilm on glass supports to remove cadmium (Macaskie and Dean, 1987; Macaskie *et al.*, 1987b). *Pseudomonas aeruginosa* has been immobilized on polyvinyl chloride films (PVC) for simultaneous denitrification and heavy metal removal. The bacterium used a component in the plastic as carbon source for growth and thereby burrowed itself into the plastic (Holló and Tóth, 1979). The use of pelleted mycelium of

filamentous fungi required no extrinsic matrix for immobilization but still bear the advantages of immobilized cells. The pelleted mycelia of *Penicillium chrysogenum* and *Rhizopus arrhizus* has been studied for metals removal in a simple contiuous flow system (de Rome and Gadd, 1991).

The appropriate choice of immobilization method and matrix used for immobilization is crucial for producing an effective biosorbent. The biosorbent in immobilized form should be of good mechanical strength and stability, highly permeable to metal ions and must cause least reduction in the metal removal capacity of the microbial cells (Macaskie and Dean, 1987; Volesky, 1987). The proportion of the matrix in the immobilized cells should be as small as possible so that the biosorptive capacity per unit weight (or volume) of biosorbent will not decrease significantly (Brierley *et al.*, 1986b; Tsezos *et al.*, 1988). It is also desirable for the matrices to be able to sorb significant amount of metals to contribute to the overall metal removal capacity of the immobilized cells (Kuhn and Pfister, 1990; Watson *et al.*, 1990). The development of such "ideal" immobilization method would require a broad spectrum of knowledge (Volesky, 1987). Actually, for some biosorbents which are promising in commercial application, the method used in immobilization has been kept secret (Brierley *et al.*, 1989; Tsezos *et al.*, 1988).

2.7.2 Recovery of Metals and Regeneration of Biosorbent

As shown earlier in Table 3, some metals are likely to get exhausted soon and the recovery of metals from metal-laden wastes has long been envisaged. The feasibility of metal recovery from biosorbent is largely governed by economic factors, for example, the price of the metals and the cost of the recovery processes. Biosorbed metals may be recovered by destructive or non-destructive methods where the biosorbent may either be destroyed to release the sorbed metals, or regenerated for further use by treating mildly with chemicals which desorb the

metals from the biosorbent. The choice of methods is principally determined by the relative costs of the two recovery processes. The metal uptake mechanism of the biosorbent also limit the choices. Intracellularly trapped metals must be recovered by breaking down the microbial cells and thus preclude the possibility of non-destructive recovery (Brierley *et al.*, 1985; Gadd, 1988).

Many investigations have been conducted to try to recover sorbed metals from the biosorbents used. The choice of eluant for metal recovery and biosorbent regeneration should be determined mainly by factors like: (1) the metal recovery efficiency of the eluant; (2) the cost of the eluant; and (3) the effect of the eluant on metal removal capacity of the biosorbent regenerated.

The recovery of uranium from biosorbent was most extensively studied. In the investigation of uranium recovery with *Rhizopus arrhizus* biomass as biosorbent, carbonate and bicarbonate were both found to be effective in elution of uranium from the biosorbent, probably because the carbonate radical was a strong complexing agent of uranium (Tsezos, 1984). It was suggested that bicarbonate was preferred to carbonate because the high pH (c.a. 10) of carbonate solution might damage the cell wall structure and operations would not be convenient at such a high pH (Tsezos, 1984). It has been demonstrated that immobilized *R. arrhizus* could maintain its biosorption capacity rather stably (after an initial drop) over 12 successive adsorption-desorption cycles using sodium bicarbonate as eluant (Tsezos *et al.*, 1989). Although mineral acids like hydrochloric acid (HCl) and nitric acid (HNO_3) eluted all the bound uranium at a concentration of 1 N, they broke the fungal mycelia into fragments. Nitric acid caused a more serious damage than HCl did probably due to its stronger oxidizing potential. Sulfuric acid (H_2SO_4) was not as effective as the above two acids in elution and it was also found to brought a more severe destruction to the mycelia than HCl did. This was attributed to the sulfate-induced structure modification of cell wall chitin, a cell wall component largely responsible for uranium binding. Ammonium sulfate was both ineffective in

uranium elution and destructive to the cell wall of *R. arrhizus* (Tsezos, 1984). In another case, a mixture of carbonate/bicarbonate solution was also found to be effective in elution of uranium bound to the biomass of *Penicillium digitatum*. The regenerated biomass showed a 100% increase in uranium removal capacity after the first extraction process (Galun *et al.*, 1983).

Other studies of metal recovery and biosorbent regeneration were far less comprehensive. Copper bound to immobilized *Saccharomyces cerevisiae* and *Trichoderma viride* could be eluted by hypochloric acid ($HClO_4$) and HCl (Townsley *et al.*, 1986; Huang *et al.*, 1990). Sulfuric acid, hydrochloric acid and nitric acid desorbed copper bound to biomass of *R. arrhizus*, *Cladosporium resinae* and *Penicillium italicum* with equal efficiencies in the pH range about 3 to 6 (de Rome and Gadd, 1987). Nitrilotriacetic acid (NTA) was also used to recover various metals bound to immobilized cells of *Zoogloea ramigera* (Kuhn and Pfister, 1989).

A method of selective metal recovery from algal biomass has been demonstrated. Copper, zinc, gold and mercury bound to immobilized cells of *Chlorella vulgaris* could be eluted selectively by sodium acetate and mercaptoethanol. Zinc followed by copper were eluted by sodium acetate at pH 2. Mercury could then be eluted by mercaptoethanol at pH 2 and finally gold was eluted at pH 5 (Darnall *et al.*, 1986).

3. Materials and Methods

3.1 Screening of Bacteria for Copper Removal Capacity

Chemicals Copper chloride ($CuCl_2.H_2O$): a stock solution containing 10 g/L of Cu(II) (0.156 M) was used in all experiments. Tris(hydroxymethyl)aminomethane (TRIS): a 10 mM TRIS buffer solution was used in all experiments described in section 3.1. pH was adjusted by concentrated hydrochloric acid (HCl) to 7.0. Diphenylcarbazide: the chemical was dissolved in 95 % ethanol to make a 10 % (w/v) solution.

Medium The screening medium (SM) was modified from Kwok (1990): D-glucose 4.0 g; NH_4Cl, 1.02 g; K_2HPO_4, 0.057 g; NaCl, 1.0 g; K_2SO_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; $CaCl_2.2H_2O$, 0.03 g; TRIS, 6.05 g; in 1 litre of distilled water. Medium's pH was adjusted to 7.5 with concentrated HCl. ($MgSO_4.7H_2O$ and $CaCl_2.2H_2O$ were added to the medium after the addition of all other constituents and pH adjustment. Glucose was autoclaved separately. No pH change was detected in the medium after autoclaving and addition of sterilized glucose solution.) Solid medium of SM was made by adding 15 g of agar while glucose, $MgSO_4.7H_2O$ and $CaCl_2.2H_2O$ were autoclaved separately. For Cu(II) containing solid medium of SM, separately autoclaved stock solution of $CuCl_2.2H_2O$ were added by appropriate volume to attain a final concentration of 5 mg/L Cu(II). Luria-Bertani (LB) medium: tryptone (Oxoid), 10 g; yeast extract (Biolife), 5 g; NaCl, 10 g; in 1 litre of distilled water. For solid LB medium, 15 g of agar was added.

3.1.1 Isolation of Bacteria from Activated Sludge

Loopfuls of activated sludge sample were added to sterile saline solution (NaCl, 0.85 %) and serially diluted to 10^{-5} with the solution. Aliquots of 0.1 mL of the dilutions were plated on LB agar and incubated at 30 °C overnight. Cultures of

bacterial isolates were maintained on LB agar plate at 4 °C and frozen at -70 °C after embedded in glycerol (final concentration 50 %).

3.1.2 Selection of Copper Resistant Bacteria from Water Samples

Wastewater samples collected from Fo Tan Nullah were added to test-tubes containing 5 mL of SM with Cu(II) at final concentration of 100 mg/L. The test-tubes were incubated at 30 °C and agitated at 200 rpm until microbial growth were observed. Aliquots of 0.1 mL of the culture were taken out, serially diluted, plated on LB agar and incubated at 30 °C overnight. Colonies of distinct morphologies were picked and inoculated again to test-tubes of SM with 100 mg/L Cu(II) to check their Cu(II) resistance. For those tubes showing cell growth, 0.1 mL aliquots were taken out, serially diluted and plated on LB agar so as to confirm purity of culture by observing colony morphologies. The Cu(II) resistant bacteria were maintained as described in section 3.1.1.

3.1.3 Pre-screening of Bacteria for Copper Uptake

Bacteria isolated from activated sludge, Cu(II) resistant bacteria selected from water samples from Fo Tan Nullah and some previously selected chromium(VI) resistant bacteria were inoculated onto SM agar plates containing 5 mg/L of Cu(II). After incubation at 30 °C overnight, the colonies were sprayed with freshly prepared diphenylcarbazide solution. After 2 minutes, the appearance of pink coloration on the colonies were scored. Those bacterial strains showing color change were chosen for further screening procedures.

3.1.4 Determination of Copper Removal Capacity of Selected Bacteria

Culture of Bacterial Cells Bacterial inoculum was prepared by inoculating single colony to SM in test-tubes, incubated at 32 °C and agitated at 200 rpm for 24 hours

to serve as inoculum. One-litre flask containing 400 mL SM was added with 0.25% inoculum, incubated at 32 °C and agitated at 200 rpm for 48 hours. The cells were then harvested by centrifugation (4,000g; 15 minutes; 4 °C), washed twice with TRIS buffer and then resuspended in 10 mL of the buffer.

Determination of Copper Removal Capacity Two millilitre of the bacterial cell suspension was added into a centrifuge bottle containing 99 mL of TRIS buffer with 50 mg/L of Cu(II). For the control experiment, 2 mL of TRIS buffer was added. After equilibration by shaking (200 rpm; 30 °C; 1 hour), the bacterial cells were removed by centrifugation (8000g, 15 minutes, 30 °C). The supernatant was decanted into a polyethylene bottle for determination of Cu(II) concentration. The dry weight of cells added was deduced by taking 2 mL of the cell suspension to a pre-weighed aluminium cup and dried at 105 °C for 24 hours. Dry weight of solutes contained in 2 mL of TRIS buffer was also determined.

Determination of Cu(II) Concentration Cu(II) concentration was determined by an atomic absorption spectrophotometer (GBC; model 902).

Calculation of Cu(II) Removal Capacity Removal capacity (RC) of Cu(II) expressed as mg Cu(II) removed per g cell dry weight and calculated by the following equation:

$$RC = \frac{(C_c - C_e)\,V}{W}$$

where C_c is the final Cu(II) concentration in the control (mg/L),
C_e is the residual Cu(II) concentration after addition of the cells (mg/L),
W is the cell dry weight (g), and
V is volume of the Cu(II)-containing buffer solution (L).

The bacterial isolate with the highest Cu(II) removal capacity was selected for further studies.

3.2 Effect of Culture Conditions on Copper Removal Capacity of *Pseudomonas putida* 5-X

Chemicals 2-(N-Morpholino)ethanesulfonic acid (MES): a buffer solution containing 10 mM MES with pH adjusted to 5.5 by potassium hydroxide was used in the following experiments. This MES buffer solution might be added with $CuCl_2$ to contain 50 mg/L Cu(II) and was designated "Cu(II) containing buffer" in the following text.

Organism The bacteria strain 5-X identified as *Pseudomonas putida* was chosen for the present study.

3.2.1 Effect of Nutrient Limitation

Media Compositions of phosphate, ammonia, sulfate, glucose limiting and nutrient non-limiting media for *Pseudomonas putida* 5-X determined by preliminary experiments are shown in Table 4. The phosphate limiting medium is the same as SM except pH. LB broth added with 6.05 g/L TRIS was also used. All media's pHs were adjusted to 8.0 with concentrated HCl. No pH change in the media was observed after autoclaving.

Culture of cells Inocula were grown in the 4 media shown in Table 4 and LB broth incubated at 32 °C for 24 hours in a shaker. Five-hundred-millilitre-flask with 100 mL of each of the 5 media was inoculated with 1 mL of inocula prepared in the corresponding media. The culture was incubated at 32 °C and shaken at 200 rpm for 48 hours. Cells were harvested by centrifugation (4000g; 10 minutes; 4 °C), washed twice with MES buffer and then resuspended in 6 mL of the buffer solution.

Determination of Copper Removal Capacity Cu(II) removal capacity of the cells was determined as described in section 3.1.4, except that MES buffer was used instead of TRIS buffer.

Table 4. Composition of phosphate, ammonia, sulfate, glucose limiting and nutrient non-limiting media for *Pseudomonas putida* 5-X

Constituents (g/L)	Media				
	Phosphate limiting	Ammonia limiting	Sulfate limiting	Glucose limiting	Nutrient non-limiting
TRIS	6.05	6.05	6.05	6.05	6.05
NH_4Cl	1.02	0.203	1.02	1.02	1.02
K_2HPO_4	0.228	1.14	1.14	1.14	1.14
NaCl	1.0	1.0	1.0	1.0	1.0
K_2SO_4	1.0	1.0	--	1.0	1.0
KCl	--	--	1.0	--	--
$MgSO_4.7H_2O$	0.5	0.5	--	0.5	0.5
$MgCl_2.6H_2O$	--	--	0.4	--	--
$CaCl_2.2H_2O$	0.03	0.03	0.03	0.03	0.03
$FeSO_4.7H_2O$	0.002	0.002	0.002	0.002	0.002
D-Glucose	4.0	4.0	4.0	1.6	4.0

3.2.2 Effect of Incubation Temperature and Culture Age

Medium The sulfate limiting medium (SLM) described in Table 4 was used.

Culture of Cells Inoculum was prepared by inoculating test-tube of 5 mL SLM with single colony from LB agar and incubating at 32 °C for 24 hours in shaker (speed 200 rpm). Five-hundred-millilitre-flask containing 100 mL SLM were added with 1 mL inocula, incubated at 32, 34 or 37 °C and shaken at 200 rpm. For each incubation temperature, cells were harvested after 12, 24, 36, 48 and 60 hours of incubation. Cells were harvested, washed and suspended in MES buffer as described in section 3.2.1.

Determination of Copper Removal Capacity Cu(II) removal capacity of the cells were determined as describe in section 3.2.1.

3.3 Determination of Copper Uptake Mechanism

Culture of Cells Bacterial cells were cultured under conditions optimal for Cu(II) removal as determined by previous experiments. These conditions are summarized in Table 5 and will be termed as "optimal conditions" in the following methods.

3.3.1 Effect of Glucose and Sodium Azide on Copper Removal Capacity

Metabolism inhibited cells were prepared by pretreatment with 1 volume in 5 mM NaN_3 solution for 1 hour. Only few colonies were recovered on LB agar plate. The cells were then washed twice with MES buffer and Cu(II) removal capacity was determined as described in section 3.2.1. Effect of glucose was investigated by suspending the cells in the Cu(II) containing buffer solution in the presence of 0.4 % (w/v) glucose (a concentration identical to that in SLM) under conditions described in section 3.2.1. Cu(II) removal capacity was determined as above.

Table 5. Culture conditions optimal for copper removal by *Pseudomonas putida* 5-X

Medium composition:	Sulfate limiting (SLM)
Incubation temperature:	37 °C
Incubation time:	36 hours

3.3.2 Transmission Electron Micrograph of *Pseudomonas putida* 5-X after Copper Uptake

Cells of *P. putida* 5-X were equilibrated with the Cu(II) containing buffer under conditions identical to the Cu(II) uptake experiment described previously. The Cu(II) loaded cells were harvested by centrifugation, dehydrated through an ethanol series and then embedded in SPURR. Sections were then examined under transmission electron microscope (Jeol; model JEM-100CXII). No fixative and stain was used in the preparation of the sections.

3.4 Effect of Pretreatment of Cells on Copper Removal Capacity of *Pseudomonas putida* 5-X

Cells cultured under optimal conditions were suspended in HCl (0.01 M) or NaOH (0.01 M) for 30 minutes, or heat treated by autoclaving for 10 minutes (121 °C; 20 atm.). Samples of pretreated cells were observed under light microscope. Cells after various pretreatments were washed twice with MES buffer and used for determination of Cu(II) removal capacity as describe in section 3.2.1.

3.5 Physico-chemical Characterization of *Pseudomonas putida* 5-X as Biosorbent for Copper Removal

3.5.1 Determination of Copper Uptake Kinetics

Cells cultured under optimal conditions were added into a plastic bottle containing 1 litre of Cu(II) containing buffer solution. The solution was kept at 30°C in a water bath and mixed by a magnetic stirrer. Samples of the cell suspension were taken out at time interval and filtered through millipore filter (pore size = 0.45 μm), with the first mL of filtrate discarded. The Cu(II) concentrations of the samples and the Cu(II) removal capacity were determined as described in section 3.2.1.

3.5.2 Determination of Freundlich Isotherm for Copper Uptake

Cells cultured under optimal conditions were added into the buffer solutions with initial Cu(II) concentrations of 2.5, 5, 10, 25 and 50 mg/L. The Cu(II) removal capacities were determined as described before. The results obtained were fitted to the linearized Freundlich equation:

$$\log q_e = \log K - 1/n \log C_e$$

where q_e is the amount of adsorbate adsorbed per unit weight of adsorbent (corresponding to Cu(II) removal capacity in this study),

C_e is the equilibrium concentration of the adsorbate, and

K and 1/n are characteristic constants

3.5.3 Effect of pH on Copper Removal Capacity

Cells of *Pseudomonas putida* 5-X cultured under optimal conditions were concentrated and suspended in 10 mM KCl solution. Two millilitres of the cell suspension was added to Cu(II) solutions at pH 3.1, 4.3, 5.5, 6.2, 7.0 and 8.0. Buffer solution containing 10 mM 3-(N-Morpholino)propanesulfonic acid (MOPS) was used to maintain pH at 7.0 and 8.0 (pH adjusted with KOH). MES buffer (10 mM) was used to maintain pH at 5.5 and 6.2. No buffer was used to maintain pH at 3.1 and 4.3 but with 10 mM KCl added instead. Final pH of the Cu(II) containing buffer solutions after Cu(II) removal were measured. Cu(II) removal capacities at the different pHs were determined as described before.

3.5.4 Effect of Metal Ions on Copper Removal Capacity

Cells cultured under optimal conditions were added to Cu(II) containing buffer solutions with equi-molar concentrations (0.78 mM) of nickel, lead or zinc {as Ni(II), Pb(II) and Zn(II) respectively in the form of chloride salts}. Buffer

solution containing only 50 mg/L Cu(II) was also used for comparison. Cu(II) Removal capacity of the cells in the presence of various metallic ions were determined as described before.

3.5.5 Effect on Anions on Copper Removal Capacity

Cells were added to Cu(II) solutions with 0.78 mM of boric acid (H_3BO_3), potassium carbonate (K_2CO_3), potassium sulfate (K_2SO_4) or 1.56 mM of potassium chloride (KCl). A buffer solution containing only 0.78 mM of $CuCl_2$ (50 mg/L Cu(II)) was used in the control experiment. Cu(II) removal capacities in the presence of various anions were determined as described before.

3.6 Copper Removal by Immobilized Cells of *Pseudomonas putida* 5-X

Chemicals Acrylamide solution (20 %): 18.2 g acrylamide and 1.8 g N,N'-methylenebisacrylamide in 100 mL distilled water. Ammonium persulfate solution (10 %; freshly prepared). N,N,N,N-tetramethylethylenediamine (TEMED).

Immobilization of Cells Six millilitres of the acrylamide solution was mixed with 9 mL of cell suspension (3.3 - 5.5 mg dry cell mL^{-1}). For the control experiment, 9 mL of MES buffer was added instead. Then 0.1 mL of ammonium persulfate solution and 0.01 mL TEMED were added and the reaction mixture was allowed to stand for 30 minutes to complete polymerization. The gel prepared was then shredded to 20 mesh and approximately 10 g of shredded gel (wet weight) was packed into a column. The column was washed with 200 mL of MES buffer before use in the Cu(II) uptake experiments.

Configuration of Bioreactor The immobilized cells was packed in a column and the Cu(II) containing buffer (50 mg Cu/L) was pumped through the column by a peristaltic pump in an upflow direction. Effluent from the column was collected with fraction volume of 10 mL by a fraction collector (Figure 2).

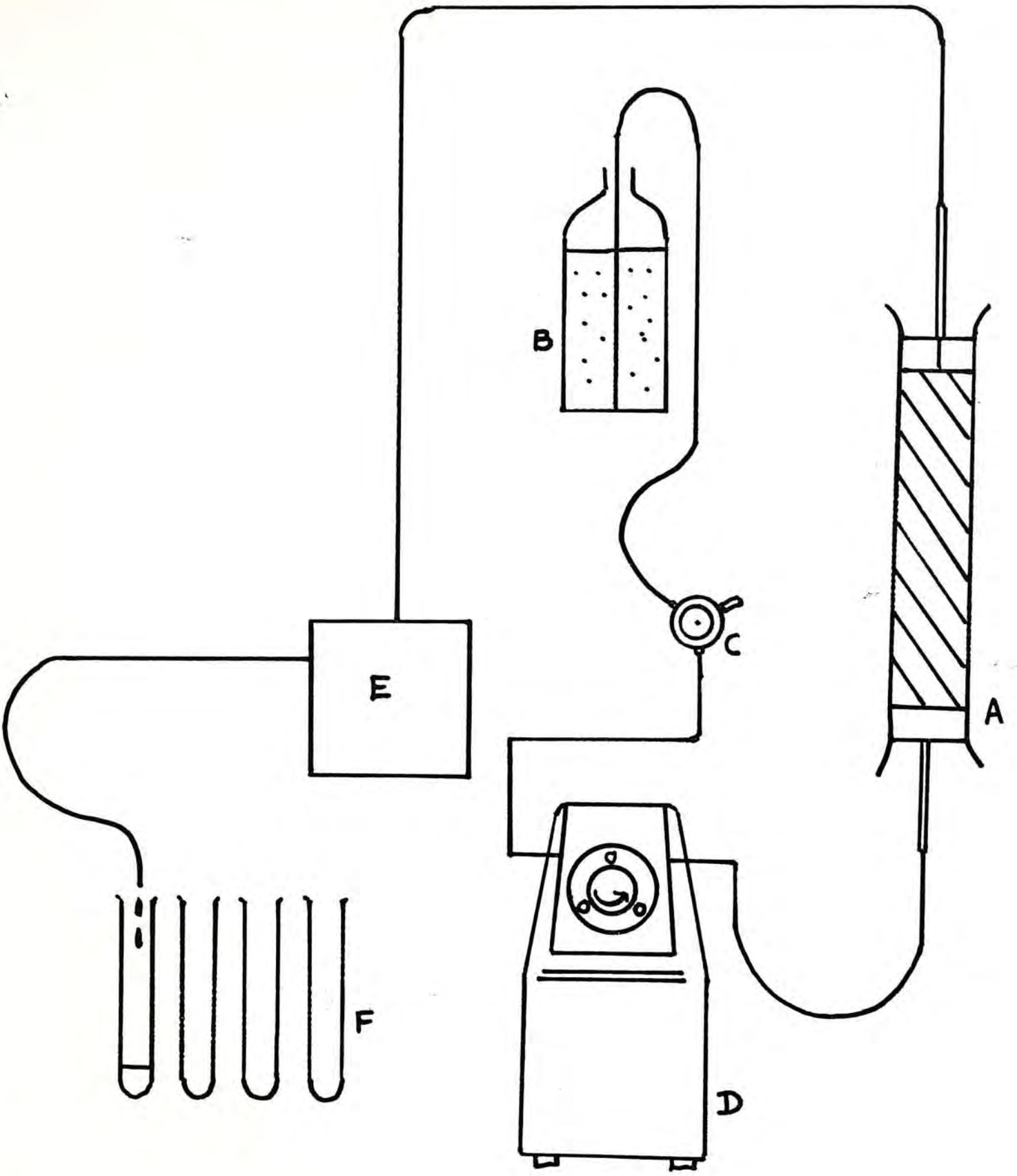

Figure 2. Configuration of bioreactor for copper removal. A: Column packed with immobilized cells. B: Reservoir of Cu(II) containing buffer solution. C: Stopcock. D: Peristaltic pump. E: Fraction collector. F: Sample collecting test-tubes.

Determination of Copper Removal Capacity Amount of Cu(II) removed by cells immobilized in polyacrylamide gel was calculated by summing up the amount of Cu(II) removed in each fraction until the effluent concentration reached 90 % of that of the untreated Cu(II) containing buffer. For the control experiment, the column was packed with polyacrylamide gel without cells. Cu(II) removal capacity of cells immobilized in gel was determined by deducting the amount of Cu(II) removed by polyacrylamide gel from that by the immobilized cells system.

3.6.1 Effect of Retention Time on Copper Removal Capacity of Immobilized Cells

Cu(II) solution was pumped through the column of immobilized cells at retention time of 1.6, 2.4, 3.1 or 4.7 minutes (corresponding with flow rate 3, 2, 1.5 and 1 mL min^{-1} respectively; the void volume of column was approximately 4.7 mL). Cu(II) removal capacities of immobilized cells at different retention times were determined as described above.

3.6.2 Efficiency of Copper Recovery from Immobilized Cells by Various Eluants

The immobilized cells were loaded with Cu(II) solution (approximately 200 mL) as described in section 3.6.1 with a retention time of 1.6 minute. Then the column was eluted with 0.1 N HCl or 0.1 M disodium salt of ethylenediamine-tetraacetic acid (EDTA; pH = 4.6) with the same retention time and the eluant was collected as 10 mL fractions by a fraction collector. The elution process terminated when no Cu(II) was detected in the eluant. The Cu(II) removal capacity of the immobilized cells was determined as described before and the amount of Cu(II) eluted by 0.1 N HCl and 0.1 M EDTA solution was determined by summing up the amount of Cu(II) eluted in each fraction. The Cu(II) recovery efficiency was expressed as the percentage of Cu(II) eluted after loading.

3.6.3 Performance of the Immobilized Cells on Multiple Copper Loading-elution Cycles

The immobilized cells were loaded with Cu(II) solution and eluted with 0.1 N HCl for 5 cycles under conditions described in section 3.6.2. The Cu(II) removal capacity and elution efficiency were determined at each cycle.

3.6.4 Treatment of Effluent from an Electroplating Factory by Immobilized Cells

A sample of effluent directly discharged from an electroplating factory was treated by the immobilized cells under conditions described in section 3.6.2. Elution of Cu(II) by HCl from the immobilized cells was also attempted.

4. Results

4.1 Screening of Bacteria for Copper Removal Capacity

The Cu(II) removal capacities of various bacteria were presented in Table 6. Some bacterial strains were found to remove Cu(II) up to 40 mg / g cell (dry weight; same for all below). Despite such high removal capacities, many of them possessed certain undesirable characteristics. Strain 4-X1 grew very slowly whlie 1-II, 3-X, 3-Y and 3-Z could not form solid pellet on centrifugation and S261A produced excess slime. Among them the strain 5-X was selected for further studies. The bacterium was identified as *Pseudomonas putida* by the API system.

4.2 Effect of Culture Conditions on Copper Removal Capacity of *Pseudomonas putida* 5-X

4.2.1 Effect of Nutrient Limitation

Figure 3 showed the effect of nutrient limitation on Cu(II) removal capacity of *P. putida* 5-X. As compared with other media, the use of sulfate limiting medium had a evident positive effect on Cu(II) removal capacity of the bacterium. Cells cultured by the ammonia, phosphate and glucose limiting media had lower removal capacities. The sulfate limiting medium was thus used in further investigation.

Table 6. Copper removal capacities of bacterial isolates from various sources.

Bacterial strain	Copper removal capacity (mg Cu / g cell dry weight)
Cu(II) resistant bacteria	
2-I	23.0
2-X	24.5
2-Y	16.2
3-II	37.0
4-I	12.5
4-X	13.2
4-Y	2.6
5-Y	2.6
Cr(VI) resistant bacteria	
1-II	37.3
1-X	28.8
1-Z2	15.8
3-I	24.5
3-X	39.0
3-Y	37.5
3-Z	36.6
4-5	33.7
4-B	30.5
4-X1	34.6
5-X	39.5
Bacteria from activated sludge	
F-2	22.9
F-3	25.7
F-5	26.6
F-6	26.4
S-1	26.6
S-3	33.4
S-6	23.9
S-7	23.8
S-8	28.3
S261A	32.0

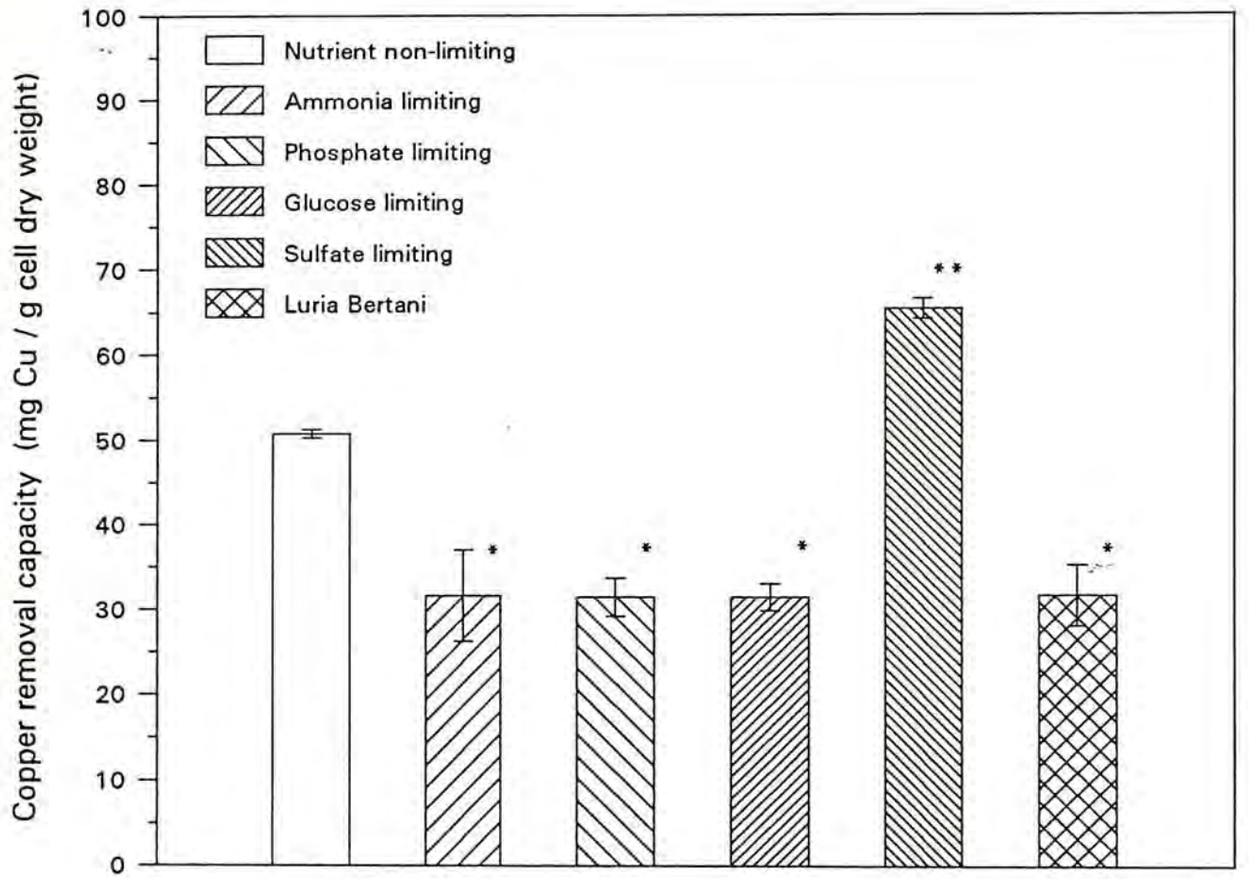

Figure 3. Effect of nutrient limitation in culture media on copper removal capacity of *Pseudomonas putida* 5-X. Each bar represents mean value of ±SEM of triplicates. Null hypothesis of no difference is tested by 1-way ANOVA ($P < 0.05$). Statistically identical groups are marked by the same number of asterisks (Tukey's test at $P = 0.05$)

4.2.2 Effect of Incubation Temperature and Culture Age

The effect of incubation temperature and culture age on Cu(II) removal capacity is presented in Figure 4. Testing of data by 2-way ANOVA indicated that both incubation temperature, culture age and the combination of the two have effect on Cu(II) removal capacity. At all three incubation temperatures, cells at late log phase of growth (12 hours of age) have their Cu(II) removal capacities uniformly at 40 mg Cu / g cell. Then the removal capacities dropped slightly when the early stationary phase was reached (24 hours of age). For incubation at 32 °C, a removal capacity about 60 mg Cu / g cell was observed at 48 hours of incubation (corresponding to late stationary phase) but the increase and decrease of incubation time by 12 hours led to decreased removal capacities. Incubation at 34 °C generally resulted removal capacities lower than 50 mg Cu / g cell. For incubation at 37 °C, removal capacity of the cells maintained at 50 - 60 mg Cu / g cell stably from 36 to 60 hours of age.

4.3 Determination of Copper Uptake Mechanism of *Pseudomonas putida* 5-X

4.3.1 Effect of Glucose and Sodium Azide on Copper Removal Capacity

Figure 5 showed the effect of the presence of glucose during Cu(II) removal and pretreatment of cells with sodium azide on Cu(II) removal capacity of *P. putida* 5-X. None of them affected Cu(II) uptake by the bacterium.

4.3.2 Transmission Electron Micrograph of *Pseudomonas putida* 5-X after Copper Uptake

Figure 6 is the electron micrograph (TEM) of cells of *P. putida* 5-X which has been exposed to Cu(II) containing buffer solution. No other stain except the Cu(II) present in the buffer was used and therefore the electron dense spots indicated where the Cu(II) was accumulated in the cell. As seen from electron micrograph, the Cu(II) taken up was mainly located at the cell surface.

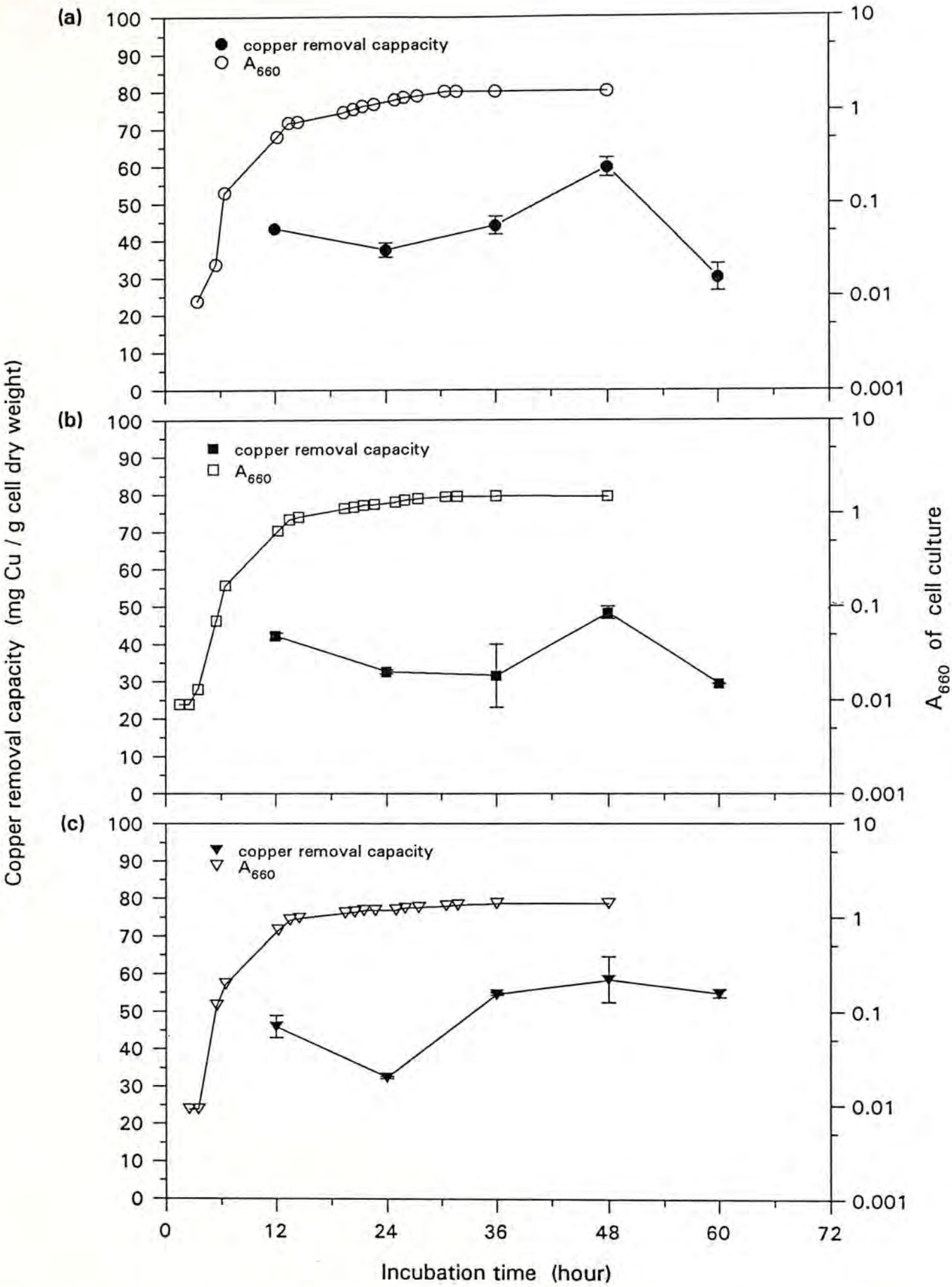

Figure 4. Effect of incubation temperature and culture age on copper removal capacity of *Pseudomonas putida* 5-X: (a) 32 °C (b) 34 °C (c) 37 °C. Each point represents mean value ±SEM of duplicates. Two-way ANOVA ($P < 0.05$) showed that both incubation temperature, culture age had effects on removal capacity and the factors interacted to affect removal capacity. Growth curve of the bacterium at each incubation temperature is also attached.

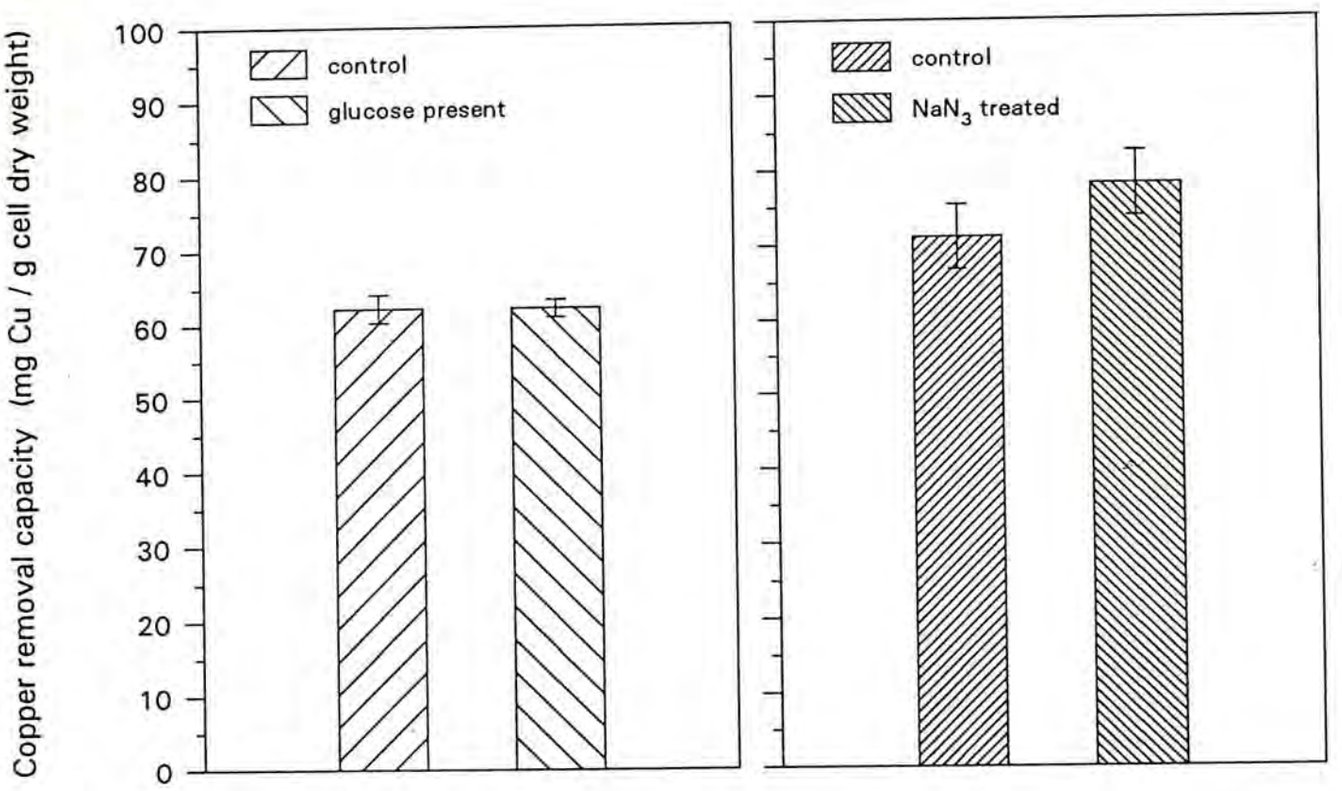

Figure 5. Effect of glucose and sodium azide on copper removal capacity of *Pseudomonas putida* 5-X. Each bar represents mean value ±SEM of triplicates. No significant difference is found between the control and treatment groups in each experiment (Student's t-test at P = 0.05).

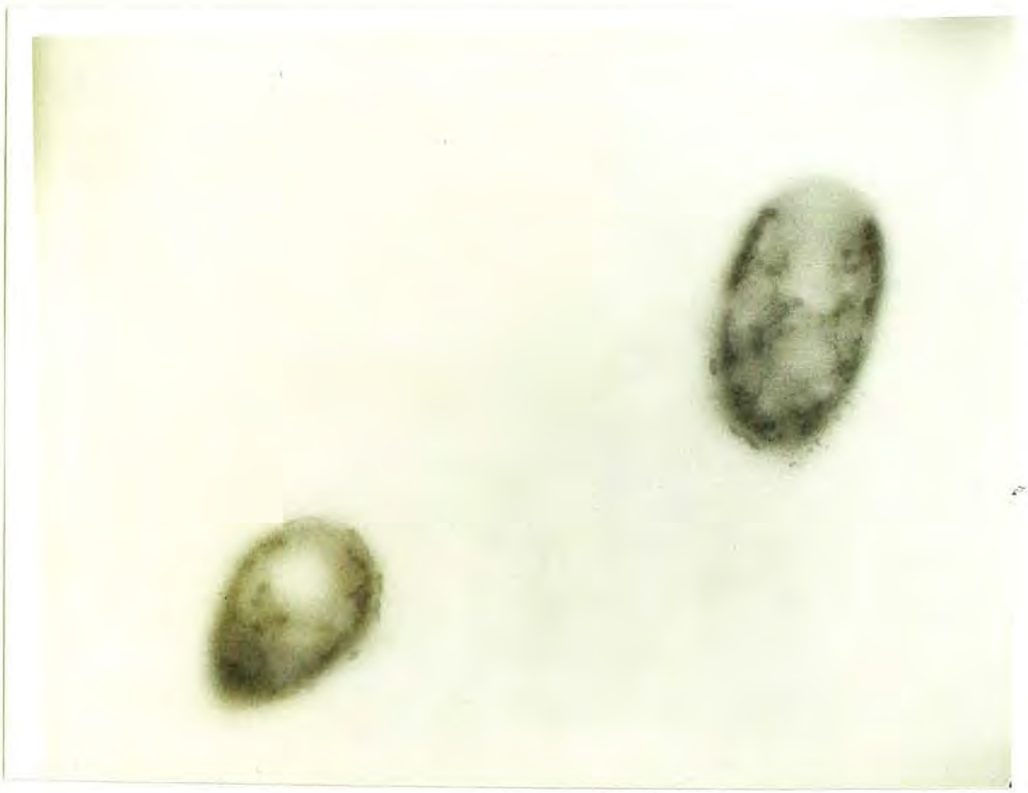

Figure 6. The transmission electron micrograph of the cells of *Pseudomonas putida* 5-X after equilibration with 50 mg/L copper. No stain was used except copper (magnification 28,000 X).

4.4 Effect of Pretreatment of Cells on Copper Removal Capacity of *Pseudomonas putida* 5-X

The effect of cell pretreatment is presented in Figure 7. It can be seen that treatment with HCl (0.01 N) and autoclaving for 5 minutes had no effect on Cu(II) removal capacity of *P. putida* 5-X. In both cases treated cells were still intact as observed under light microscope. Treatment with KOH (0.01 M) and autoclaving for 10 minutes brought a great loss of removal capacity. Cell lysis was observed on treatment with KOH. Cells autoclaved for 10 minutes shrank and clumped together as observed under light microscope.

4.5 Physico-chemical Characterization of *Pseudomonas putida* 5-X as Biosorbent for Copper Removal

4.5.1 Determination of Copper Uptake Kinetics

Figure 8 represents the Cu(II) uptake kinetic of *P. putida* 5-X. It was shown that Cu(II) removal started with a very rapidly uptake process within the first 10 minutes followed by a stage of slow uptake lasting for about 90 minutes. Over half of the total Cu(II) taken up by the bacterial cells was removed within the first 10 minutes.

4.5.2 Determination of Freundlich Isotherm for Copper Uptake

Figure 9 is a linearized Freundlich plot of Cu(II) adsorption isotherm. The regression analysis showed that Cu(II) adsorption process by *P. putida* 5-X conformed well to the Freundlich equation ($\log q_e = 0.939 - 0.503 \log C_e$; $r^2 = 0.939$).

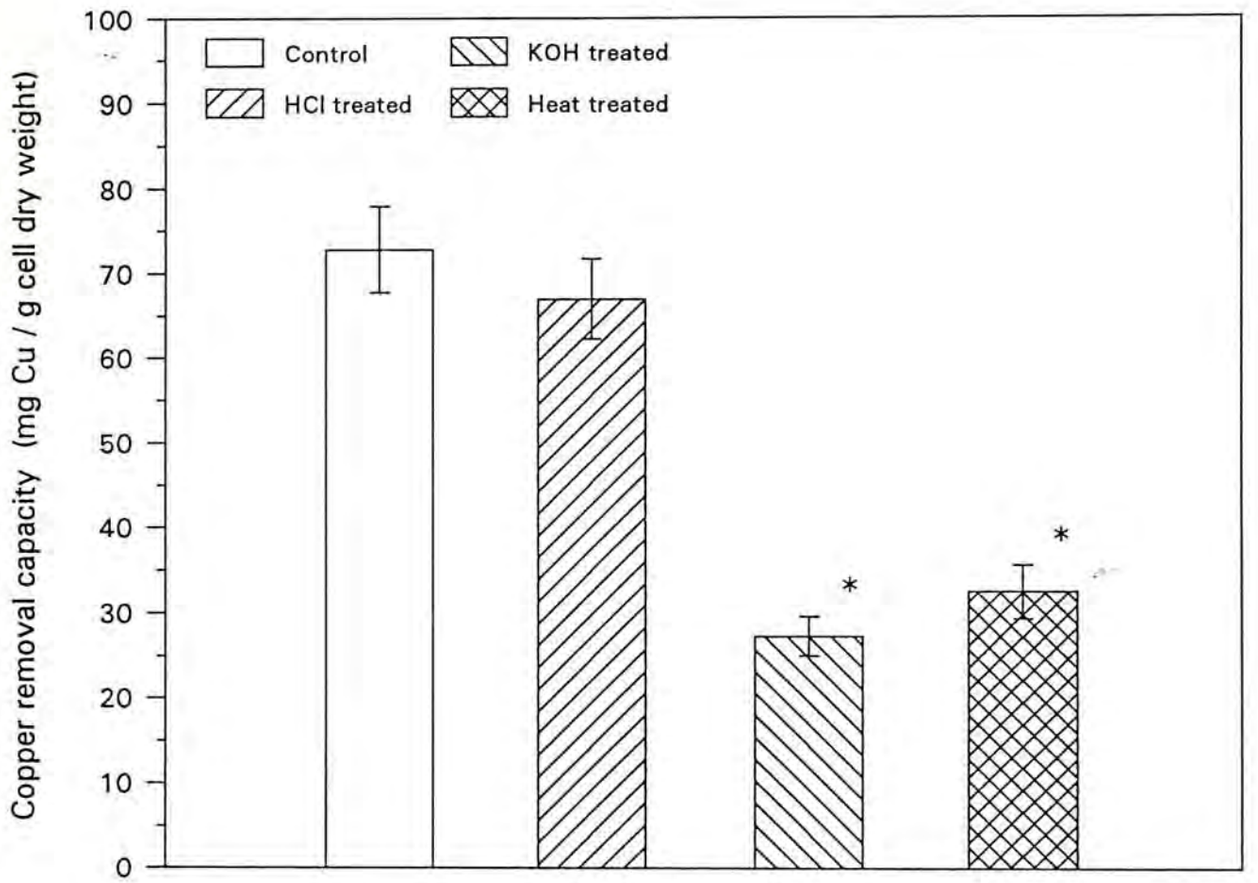

Figure 7. Effect of cell pretreatments on copper removal capacity of *Pseudomonas putida* 5-X. Each bar represents mean value ±SEM of triplicates. Null hypothesis of no difference is tested by 1-way ANOVA. Statistically identical groups are marked by the same number of asterisks (Tukey's test at P = 0.05).

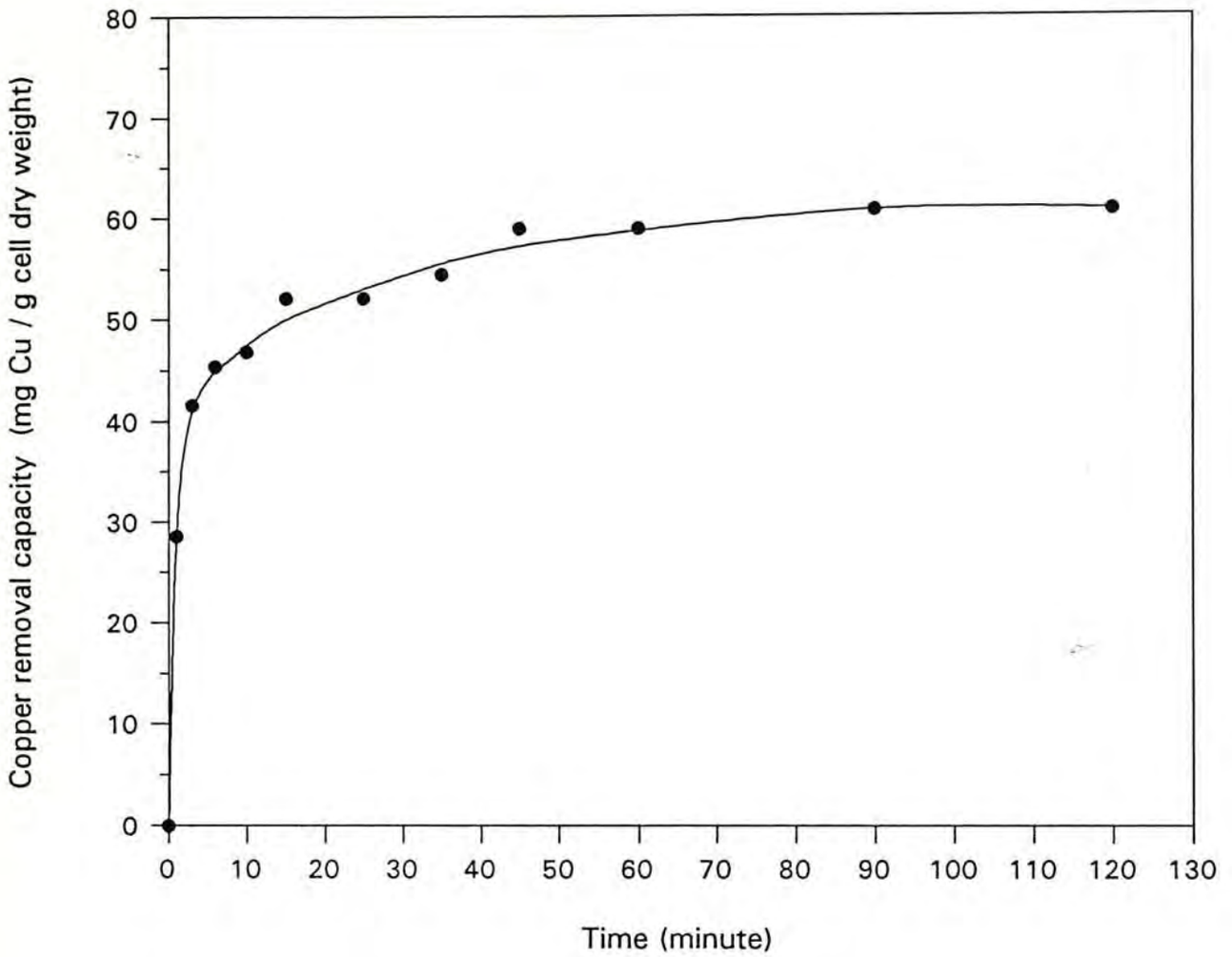

Figure 8. The copper uptake kinetics of *Pseudomonas putida* 5-X. The graph shows typical result of one of the two sets of experiments.

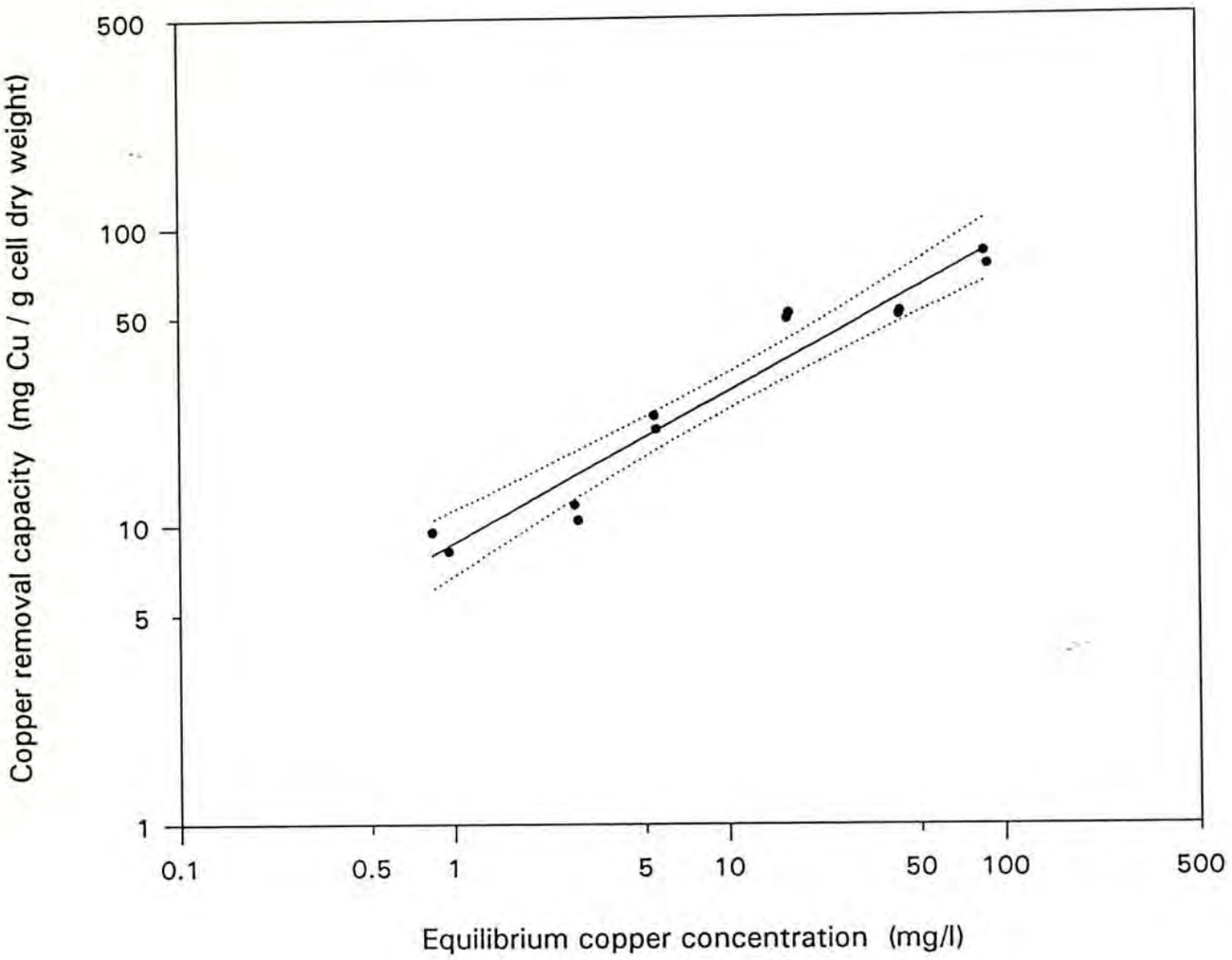

Figure 9. The linearized Freundlich isotherm for copper uptake by *Pseudomonas putida* 5-X at 30 °C as represented by the regression line. ($r^2 = 0.939$; Freundlich equation is determined to be $\log q_e = 0.939 - 0.503 \log C_e$). Dotted lines show 95 % confidence interval.

4.5.3 Effect of pH on Copper Removal Capacity

As shown in Figure 10, there was a general increase in Cu(II) removal capacity in accordance with an increase in pH. The increase was very pronounced at pH 7 - 8, up to almost a five-fold of enhancement as compared to the removal capacity at pH 5.5. The removal capacity at pH 3.1 was similar to that at pH 4.3.

4.5.4 Effect of Metal Ions on Copper Removal Capacity

Figure 11 presented the effect of various metal ions on Cu(II) removal capacity of *P. putida* 5-X. Ni(II) did not affect Cu(II) uptake while Zn(II) inhibited it slightly. Pb(II) reduced the Cu(II) removal capacity by about 5 fold.

4.5.5 Effect of Anions on Copper Removal Capacity

Figure 12 presented the effects of various anions on Cu(II) removal capacity of *P. putida* 5-X. The presence of carbonate ion did not inhibit Cu(II) uptake. Borate ion, sulfate ion and chloride ions slightly inhibited Cu(II) uptake to the similar extent.

4.6 Copper Removal by Immobilized Cells of *Pseudomonas putida* 5-X

4.6.1 Copper Removal Capacity of Immobilized Cells and Breakthrough Curve for Copper Removal

The Cu(II) removal capacity of the immobilized cells was 38.3 mg Cu / g cell $\pm$ 0.15 (SEM). Figure 13 showed the breakthrough curve of Cu(II) removal by immobilized cells of *P. putida* 5-X. As shown by the curve, the effluent Cu(II) concentration increased quickly with elution volume but it did not reached the initial Cu(II) concentration for prolonged perfusion of the Cu(II) containing buffer.

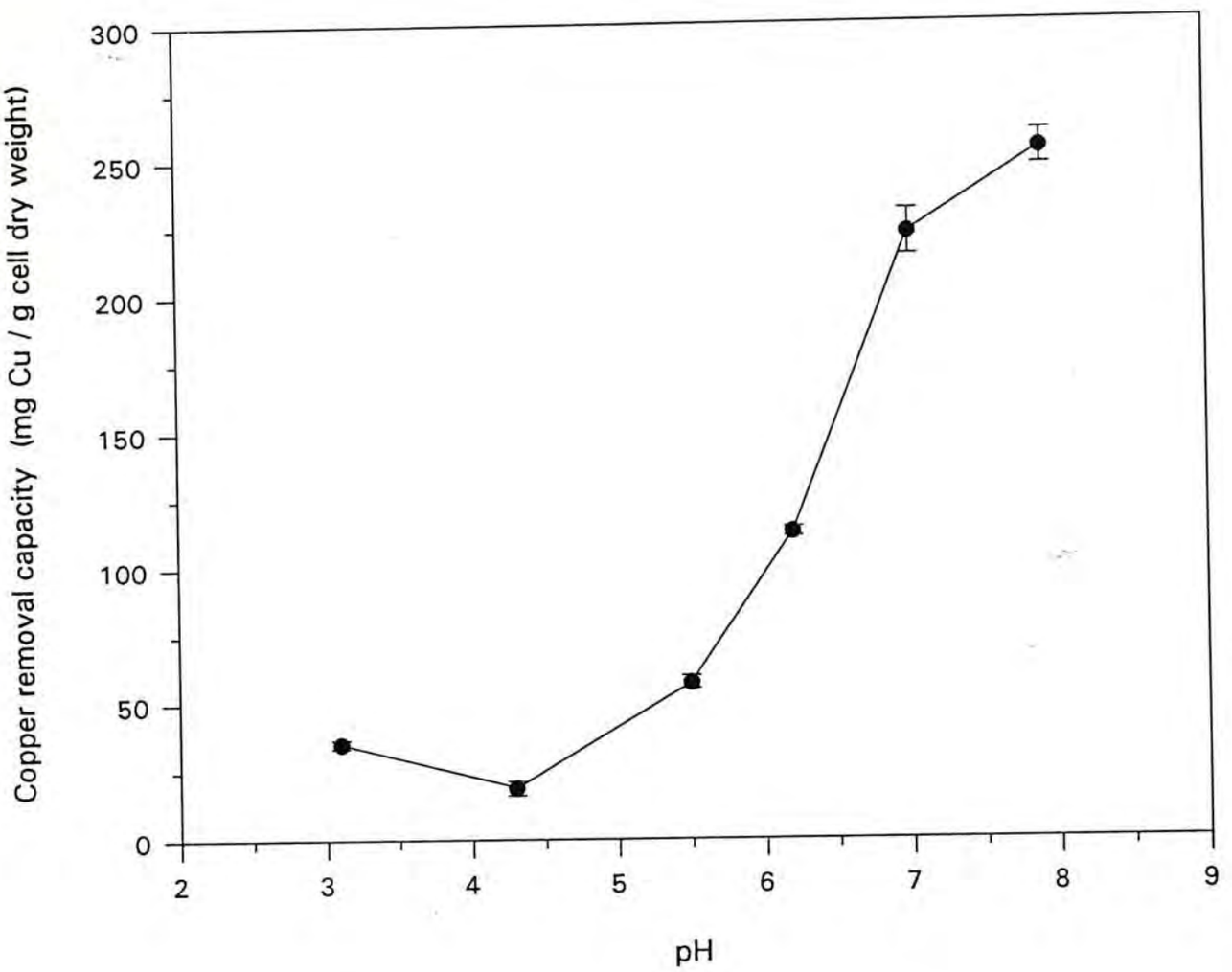

Figure 10. Effect of pH on copper removal capacity of *Pseudomonas putida* 5-X. Each point represents mean value ±SEM of triplicates.

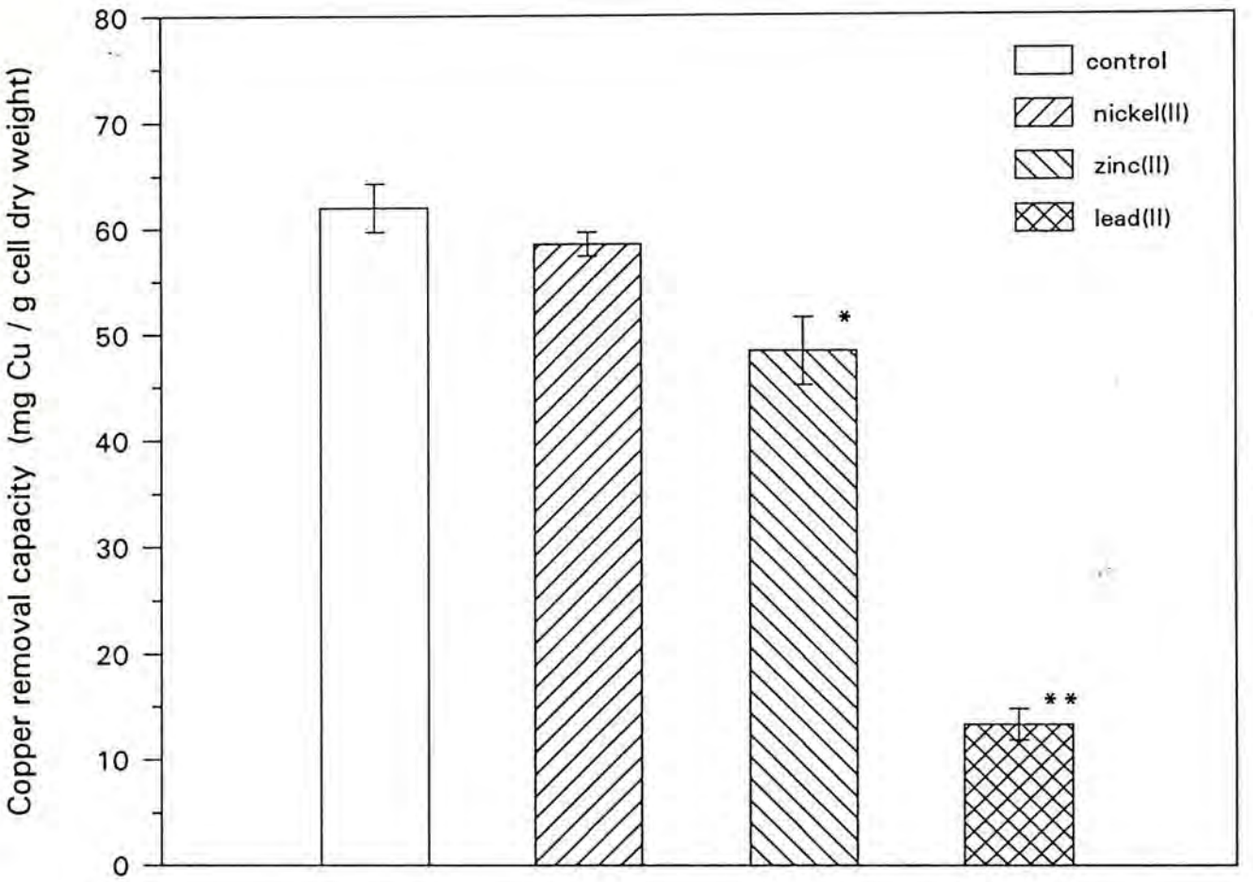

Figure 11. Effect of metal ions on copper removal capacity of *Pseudomonas putida* 5-X. Each bar represents mean value ±SEM of triplicates. Null hypothesis of no difference is tested by 1-way ANOVA. Statistically identical groups are represented by the same number of asterisks (Tukey's test at P = 0.05).

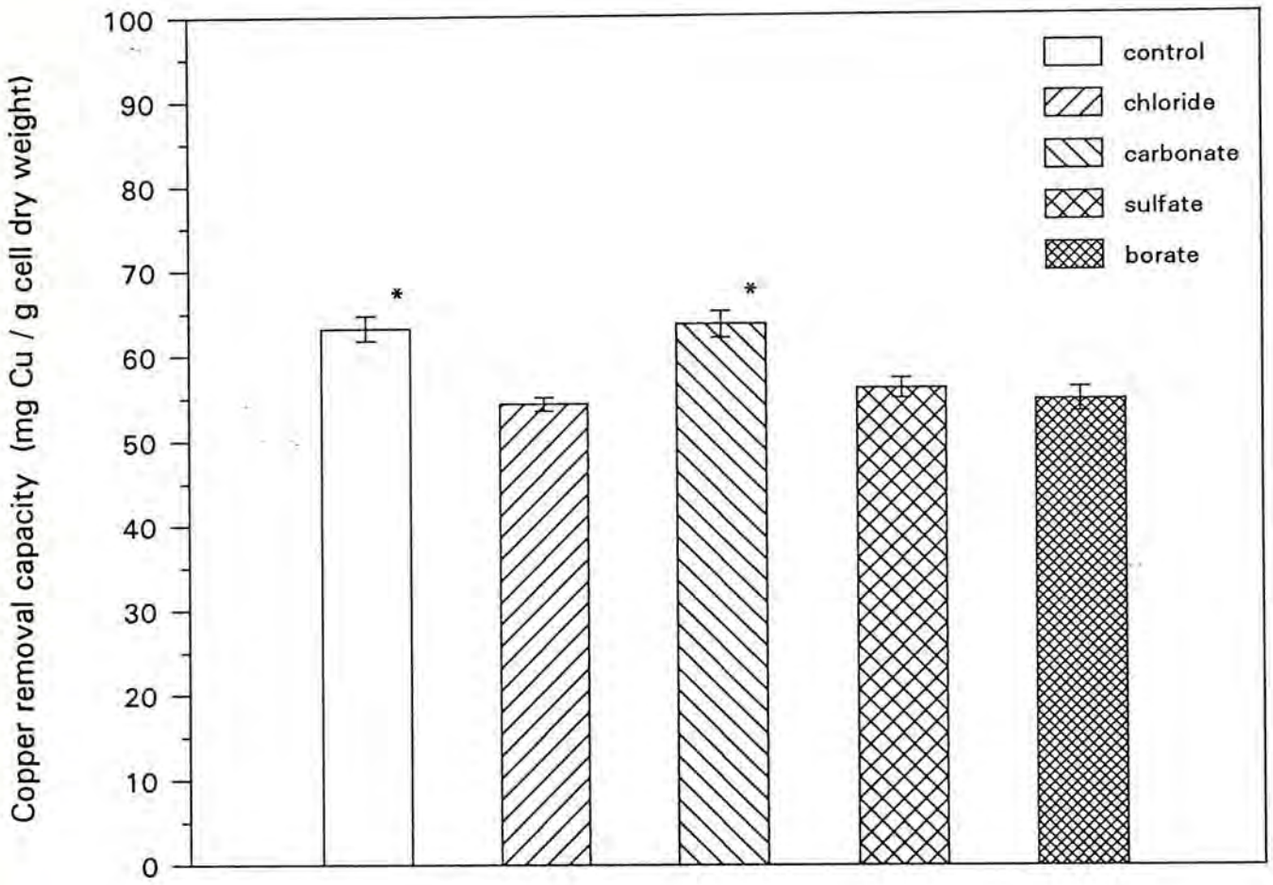

Figure 12. Effect of anions on copper removal capacity of *Pseudomonas putida* 5-X. Each bar represents mean value ±SEM of triplicates. Null hypothesis of no difference is tested by 1-way ANOVA. Statistically identical groups are marked by the same number of asterisks (Tukey's test at P = 0.05).

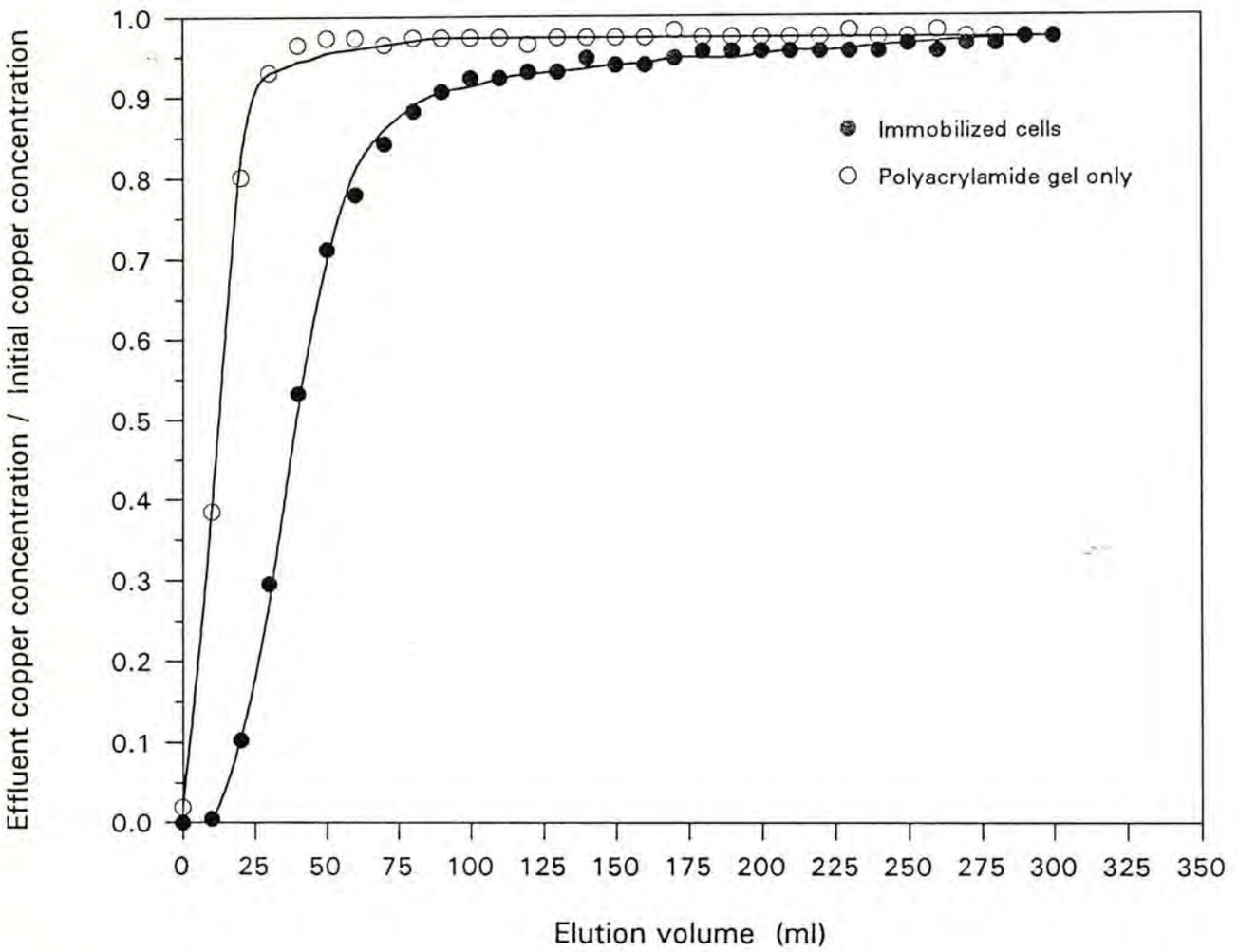

Figure 13. The breakthrough curve for copper removal by immobilized cells of *Pseudomonas putida* 5-X and polyacrylamide gel only. The graph shows typical result of one of two sets of experiments.

4.6.2 Effect of Retention Time on Copper Removal Capacity of Immobilized Cells

Figure 14 showed that generally the longer the retention time, the greater the removal capacity of the immobilized cells. However, the overall effect was not prominent and when the retention time was over 3 minutes no further increase was found.

4.6.3 Efficiency of Copper Recovery from Immobilized Cells by Various Eluants

The results in Table 7 showed that HCl and EDTA were of similar efficiency in Cu(II) recovery from the immobilized cells. None of them recovered 100 % of the Cu(II) removed. The cells remained intact after contacting with both eluants as observed under light microscope. In the following experiments involving Cu(II) recovery and column regeneration, 0.1 N HCl was used as regenerant.

4.6.4 Performance of Immobilized Cells on Multiple Copper Loading-elution Cycles

As shown in Figure 15, after the first elution by HCl the Cu(II) removal capacity of the immobilized cells doubled at the second Cu(II) loading. The removal capacity of the immobilized cells decreased again on further loading-elution cycles of the column. However, the removal capacities of the immobilized cells after first elution did not dropped below the initial removal capacity all through the other four cycles.

4.6.5 Treatment of Effluent from an Electroplating Factory by Immobilized Cells

The heavy metals composition of the effluent sample collected from an electroplating factory was shown in Table 8. The Cu(II) removal capacity and percentage recovery of Cu(II) were presented in Table 9. The removal capacity of

the immobilized cells was 24.6 mg Cu / g cell. Over 90 % of the removed Cu(II) was recovered by 0.1 N HCl.

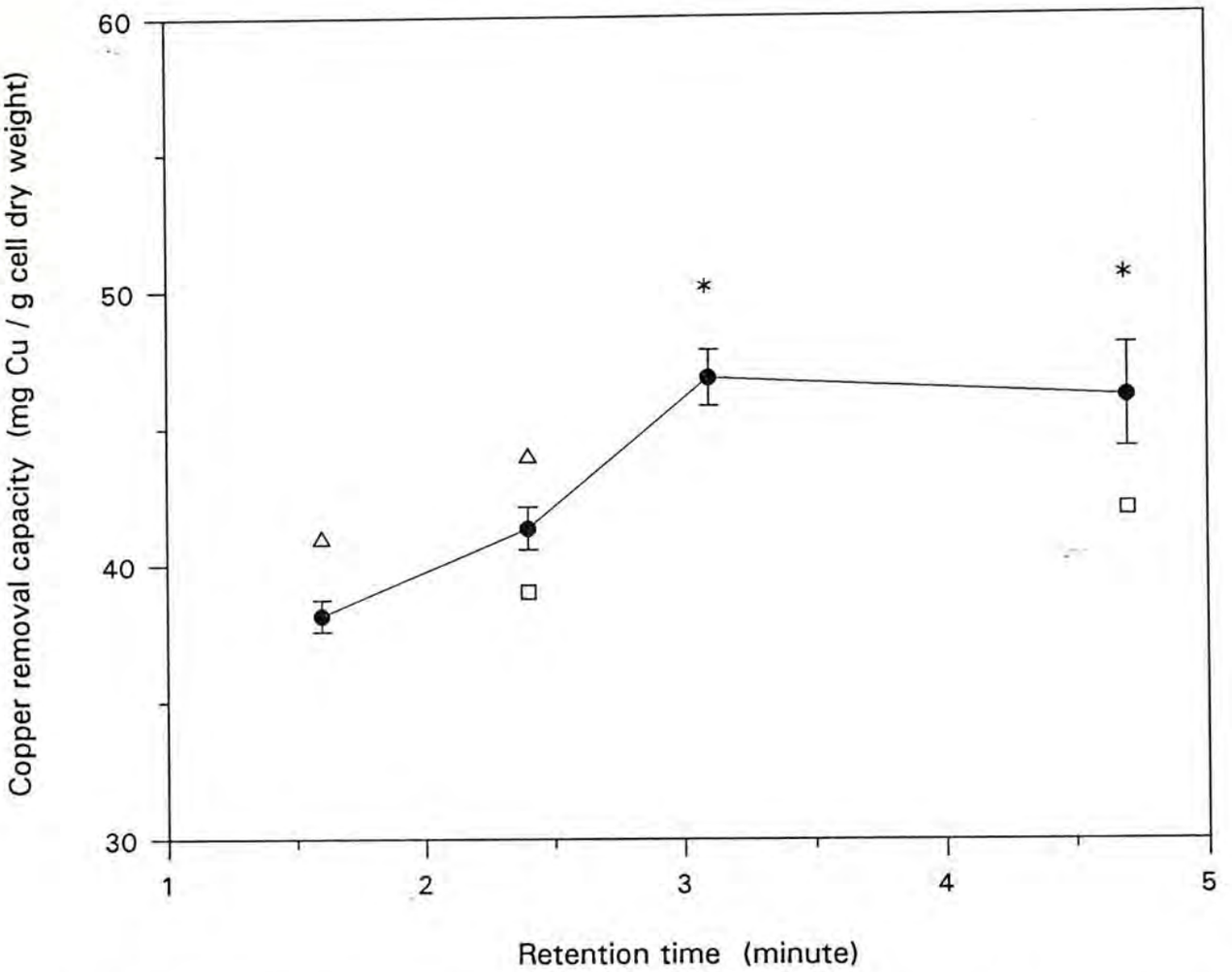

Figure 14. Effect of retention time on copper removal capacity of immobilized cells of *Pseudomonas putida* 5-X. Each point represents mean value ±SEM of triplicates. Null hypothesis of no difference is tested by 1-way ANOVA. Statistically identical groups are marked by the same number of asterisk, triangle or square (Tukey's test at P = 0.05).

Table 7. The percentage recovery of copper from loaded immobilized cells by 0.1 N HCl and 0.1 M EDTA solution

Eluant	% recovery	SEM
HCl	93.2*	±6.2
EDTA	88.8*	±1.9

*Results represent mean values of duplicates ±SEM. No significant difference is found between the two groups (Student's t-test at P = 0.05).

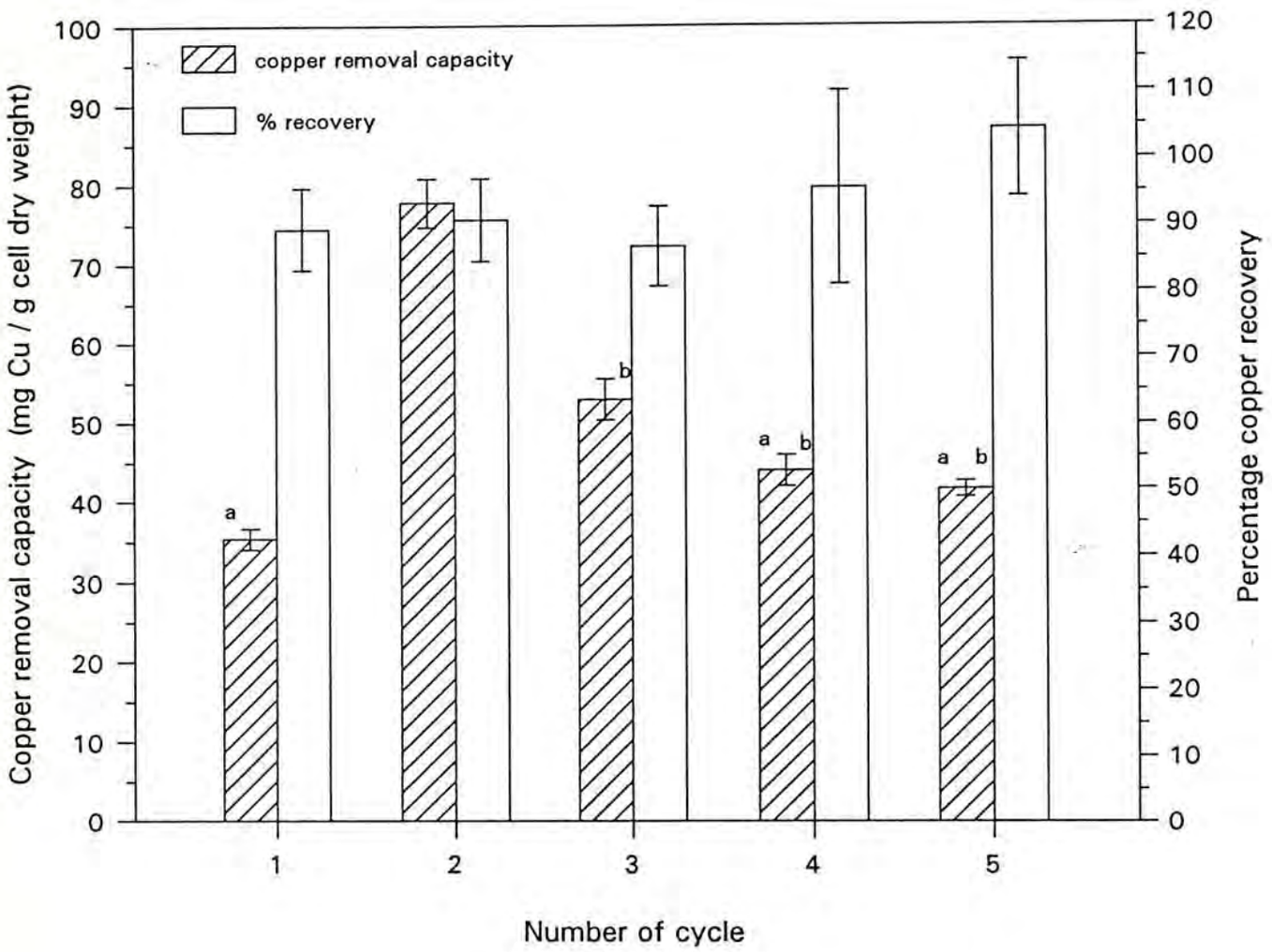

Figure 15. Copper removal capacities and percentage recovery of copper by immobilized cells of *Pseudomonas putida* 5-X for 5 loading-elution cycles. Each bar represents mean value ±SEM of duplicates. Null hypothesis of no difference is tested by 1-way ANOVA. Statistically identical groups are designated "a" or "b". For percentage recovery of copper, no significant difference was found at different elution cycles (Tukey's test at $P = 0.05$).

Table 8. Heavy metal composition of the effluent sample collected from an electroplating factory[1]

Metal	copper	chromium	lead	nickel	zinc
Concentration (mg/L)	16.96	0.09	ud[2]	34.12	1.21

[1]pH of the sample was 6.1

[2]ud=undetectable

Table 9. Copper removal and recovery from effluent sample collected from an electroplating factory by immobilized cells of *Pseudomonas putida* 5-X

Cu(II) removal capacity (mg Cu / g cell)	24.6
Percentage recovery of Cu(II)	96.7

5. Discussion

5.1 Screening of Bacteria for Copper Removal Capacity

In the screening of heavy metals accumulating microorganisms, resistance to heavy metals was usually expected to correlate with the microorganisms' extra-ordinary capacity to accumulate the metals of interest. Therefore, some screening programs focused on microorganisms resistant to the metals of interest (Baldry and Dean, 1980; Coleman and Paran, 1983; Wnorowski, 1991). However, in the results of these works reported, such correlation seemed not to be evident. Direct selection of heavy metals accumulating microorganisms have been achieved by using radioactive isotopes of the metals of interest. Autoradiographs of the microorganisms growing on the radioactive isotope containing plate indicate metal accumulating microorganisms by the presence of dark spots on the X-ray film (Zamani *et al.*, 1985). However, the method is expensive and requires additional safety measures.

The pre-screening method used in the present study also sought to select for heavy metal accumulating microorganisms directly by detecting the presence of Cu(II) on the cells. Diphenylcarbazide (DPC) is a reagent routinely used in colorimetric analysis of Cr(VI) and Cu(II) (Marczenko, 1986). It reacts with Cu(II) to form a color complex with a maximum absorbance at 495 nm. In the present study, it was found that bacterial cells growing on solid medium containing Cu(II) turned pink on addition of DPC and no such color change was observed when the bacteria were grown on medium without Cu(II). Here, it was hypothesized that the rate of coloration and intensity of color were proportional to the Cu(II) removal capacity of the microorganisms added with DPC. As shown by the results in Table 6, most of the bacteria selected by this method could remove Cu(II) by removal capacities over 20 mg Cu / mg cell. Although the use of DPC in pre-screening of bacteria was not an established application, it seemed that this method was really

useful in the present study.

However, the correlation between color change and Cu(II) removal capacity remained hypothetical and had not been statistically established in the present study. Actually, the rate of color development of the DPC-Cu(II) complex changed greatly by pH (Turkington and Tracy, 1958). Some other bacterial cell surface properties in addition to amount of Cu(II) accumulated on the cell surface could have affected the chromogenic reaction and rendered the use of this method invalid. But paradoxically, in the present investigation the effort to be used in such an establishment of correlation would exceed that involved in a screening program without the pre-screening steps. Probably, the approach of using colorimetric reagents for rapid pre-screening would be meaningful if the screening program involves a very large number of microorganisms which justifies the effort used in the establishment of correlation between color intensity and Cu(II) removal capacity.

5.2 Effect of Culture Conditions on Copper Removal Capacity of *Pseudomonas putida* 5-X

5.2.1 Effect of Nutrient Limitation

Sulfate limiting medium was found to have an positive effect on Cu(II) removal capacity of the *P. putida* 5-X. As discussed in section 2.6.1, composition of culture medium used affected cell surface properties of bacteria and thus the metal removal capacity of the bacterial cells. Kwok (1990) also found that nutrient limitation could affect the nickel(II) removal capacity of an *Enterobacter* sp. However, what specific changes were induced in cells of *P. putida* 5-X by sulfate limitation were yet to be determined. A few reports described physiological and morphological changes of *Pseudomonas* spp. induced by limitation of various nutrients (McKellar and Cholette, 1984; Azegami *et al.*, 1988; Persson *et al.*, 1990)

but the effect of sulfate limitation was not investigated.

5.2.2 Effect of Incubation Temperature and Culture Age

It was found that both incubation temperature and culture age had effects on Cu(II) removal capacity of *Pseudomonas putida* 5-X and the two factors interact to affect the Cu(II) removal capacity of the bacterium. However, No relevant information from other sources on physiological changes associated with incubation temperature and culture age could throw any light on these effects and the responsible changes associated with this two factors awaited investigation.

Culture incubated at 37 °C seemed to be optimal for *P. putida* 5-X in Cu(II) removal. The removal capacity after 36 hours of incubation was high and stably maintained for at least 24 hours of further incubation. Although incubation at 32 °C for 48 hours resulted a similarly high removal capacity, the incubation time required was longer than that at 37 °C. Furthermore, at 32 °C of incubation any increase or decrease of culture age could affect the removal capacity tremendously, implying the need for stringent control of culture conditions, which would be unfavorable in the production of the bacterial cell mass. Incubation at 34 °C produced cells with generally low removal capacities. Thus, incubation at 37 °C for 36 hours was the condition chosen for cell culture in other experiments.

Culture conditions which can enhance Cu(II) removal capacity of *P. putida* 5-X has been devised by the above experiments. Anyway, the investigated conditions are by no means exhaustive and a lot more manipulations are possible. Glucose-mineral salts media have been used in the present study because their nature are more defined than complex media and are more suitable for laboratory studies. However the possibility of use of low-cost substrates for cell culture should also be attempted because low production cost of cell mass is crucial for the produced biosorbent to be commercially viable.

In the following discussion, the term "optimal conditions" is referred to culturing the cells of *P. putida* 5-X in the sulfate limiting medium at 37 °C for 36 hours.

5.3 Determination of Copper Uptake Mechanism of *Pseudomonas putida* 5-X

5.3.1 Effect of Glucose and Sodium Azide on Copper Removal Capacity

The presence of glucose during Cu(II) removal and pretreatment of cells with sodium azide did not affect Cu(II) removal capacity of *P. putida* 5-X. Glucose was used as an energy source for metabolism while sodium azide was used as a metabolism inhibitor (Townsley *et al.*, 1986). The results showed that the Cu(II) uptake by *P. putida* 5-X was probably metabolism-independent. This implied that the bacterial cells might not lose its Cu(II) removal capacity even though killed by any toxic constituents contained in the metal-laden wastewaters.

5.3.2 Transmission Electron Micrograph of *Pseudomonas putida* 5-X after Copper Uptake

Since no stain was used except Cu(II) in the preparation of the section, the opaque regions were supposed to be where Cu(II) was located in the cells of *Pseudomonas putida* 5-X. However, the identity of the electron dense substances should be confirmed by the use of energy-dispersive X-ray analysis.

The transmission electron micrograph showed that the Cu(II) accumulated by the cells of *Pseudomonas putida* 5-X was largely located at the cell surface in a slightly dispersed form. Some Cu(II) is trapped inside the cell. This observation implied that Cu(II) uptake by *P. putida* 5-X was mostly by metabolism-independent binding on to the cell surface. This was consistent with the results obtained which indicated that Cu(II) uptake by the bacterium was metabolism-independent and most of the bound Cu(II) could be non-destructively recovered by simple elution process

(see sections 4.3.1 and 4.6.3).

However, the preparation method of the sample was not satisfactory since many interferences were brought to the bacterial cells through the ethanol-dehydration and embedment processes. Therefore, the electron micrograph should be interpreted with caution. A better method for electron micrograph preparation would be using scanning transmission electron microscopy in which fixation, dehydration and embedment of specimen were not necessary for the purpose of this experiment (Aiking *et al.*, 1984; Macaskie *et al.*, 1987a).

5.4 Effect of Pretreatment of Cells on Copper Removal Capacity of *Pseudomonas putida* 5-X

The effect of cell pretreatment on Cu(II) removal capacity was studied to see whether any of these treatments could enhance the removal capacity of *P. putida* 5-X significantly. Treatment with HCl had no effect on its Cu(II) removal capacity. Although no augmentation of removal capacity was found, this also implied that the cells of *P. putida* 5-X might tolerate exposure to acidic environment without loss of removal capacity in subsequent Cu(II) removal. However, structural changes of the HCl treated cells was revealed by the change of cell suspension's color from pink to white and the reduction of packed cell volume.

In contrast to cases reported previously in which alkali treatment enhance heavy metal uptake by microorganisms (see section 2.6.2), treatment with alkali (as 0.01 M KOH) did not enhance Cu(II) removal capacity of *P. putida* 5-X but reduced it significantly. Cell lysis was also observed after the treatment. The contradictory effects might be due to structural differences in cell envelopes of different groups of microorganisms. Cases reported previously involved fungi and a Gram-positive bacterium. Fungal cell wall is made up of microfibrils of chitin or cellulose embedded in an amorphous matrix (Gooday and Trinci, 1980) while Gram-positive bacteria possess a thick peptidoglycan wall (Shockman and Barrett,

1983). Their cell wall might be more resistant to alkali treatment. On the other hand, Gram-negative bacteria possess only a thin peptidoglycan sacculus surrounded by an outer membrane. As *P. putida* 5-X was a Gram-negative bacterium, its cell envelope might be more susceptible to alkali treatment. Thus, cell lysis occurred and affected Cu(II) removal capacity of the bacterium.

Heat treatment by autoclaving for 10 minutes reduced Cu(II) removal capacity of *P. putida* 5-X largely. The outermost layer of Gram-negative bacteria was the outer membrane and one of its components are proteins which are generally susceptible to high temperature. If these proteins were responsible for Cu(II) binding, the removal capacity of the cells would be considerably affected. Moreover, as observed under light microscope, the cells autoclaved for 10 minutes shrank and clumped together.

As no pretreatment method gave any enhancement of Cu(II) removal capacity, none of them was used in the later experiments.

5.5 Physico-chemical Characterization of *Pseudomonas putida* 5-X as Biosorbent for Copper Removal

5.5.1 Copper Uptake Kinetics

The Cu(II) uptake kinetics of *P. putida* 5-X conformed to the general pattern described previously (section 2.5). However, as mentioned above, Cu(II) uptake by the bacterium was metabolism-independent. The stage of slow uptake could be due to diffusion of Cu(II) into the bacterial cells instead of active uptake (Huang *et al.*, 1990). The uptake kinetics indicated that *P. putida* 5-X could remove Cu(II) very quickly if applied in treatment of industrial effluents.

5.5.2 Freundlich Isotherm for Copper Uptake

The Freundlich isotherm is an empirical expression that encompasses the heterogeneity of adsorbent surface and the exponential distribution of adsorption sites energies. No physical significance has been successfully attached to the parameters K and 1/n. However, K can be taken as a relative indicator of adsorption capacity while 1/n is indicative of the intensity of the adsorption reaction (Weber, 1985). Despite the lack of theoretical basis of Freundlich equation, it usually describes adsorption equilibrium data better than the other adsorption models with sound theoretical basis.

The Freundlich plot for Cu(II) uptake by *P. putida* 5-X fitted the Freundlich isotherm well, implying that Cu(II) uptake by the bacterium was largely an adsorption process. As shown by the isotherm, a residual Cu(II) concentration below 1 mg/l could be attained and this showed that *P. putida* 5-X fulfilled the requirement of good biosorbent (see section 2.4.3).

Adsorption isotherms are useful in the description of adsorption behavior of particular adsorbate-adsorbent systems over a range of equilibrium concentration of the adsorbate at a particular temperature. However, the relation of removal capacity and equilibrium concentration of Cu(II) might be true only within the studied concentration range and extrapolation of the isotherm should be avoided, especially for adsorbents as heterogeneous in nature as biosorbent.

5.5.3 Effect of pH on Copper Removal Capacity

At low pH the competition of hydrogen ions with Cu(II) ions for binding sites might have caused the decreased Cu(II) removal capacity of *P. putida* 5-X. The rise in removal capacity at pH higher than 5.5 was probably due to the precipitation of Cu(II). At pH 6.2, Cu(II) precipitate was not visible but at pHs above 7.0, sedimentation of the precipitate was observed. The precipitate formed

might deposit on the cells to result a large increase in Cu(II) removal capacity.

The results obtained here were not consistent with those found in the case of Cu(II) uptake by native and acid treated cells of *Saccharomyces cerevisiae* (Huang *et al.*, 1990), where an optimal pH for Cu(II) uptake was found to be 5.5. The drop of Cu(II) uptake was attributed to the complexation of Cu(II) with cell wall proteins solubilized on exposure to Cu(II) ions. While no such drop of Cu(II) uptake at pH higher than 5.5 was observed in *P. putida* 5-X, the problem of protein solubilization might be negligible in the bacterium. However, such difference could also be due to the specific mechanisms involved in Cu(II) uptake by the two microorganisms and variations in experimental conditions such as initial Cu(II) concentration and cell mass concentration.

5.5.4 Effect of Metal Ions on Copper Removal Capacity

Figure 11 showed that the three metal ions tested inhibited Cu(II) uptake by *P. putida* 5-X in a decreasing order of Pb(II) > Zn(II) > Ni(II). This observation conformed to the Irving-Williams series which describes the relative stability of complexes formation of divalent metal ions with a given ligand: $Ba^{2+} < Sr^{2+} < Ca^{2+} < Mg^{2+} < Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$ (Huheey, 1983). As shown by the series, both Ni(II) and Zn(II) form less stable complexes with ligands than Cu(II) do. The presence of Pb(II) reduced the Cu(II) removal capacity by 5 folds. The strong inhibition might be due to its high electronegativity which implied strong association with ligands (the electronegativity of lead is 2.33 and that of copper is 1.90). Actually, Pb(II) generally shows a very high affinity to organic ligands such as humic acids in the natural environment (Tyler *et al.*, 1989). As both Ni(II) and Zn(II) are commonly found in electroplating effluents in Hong Kong, the present result indicated that *P. putida* 5-X can remove Cu(II) from these effluents with little interference from these metal ions.

5.5.5 Effect of Anions on Copper Removal Capacity

The studied anions commonly occurred in plating baths used in the electroplating factories in Hong Kong (Chiu *et al.*, 1987) and apparently they had little effect on Cu(II) removal capacity of *P. putida* 5-X. However, a large excess of these ions might produce more pronounced effect (Faison *et al.*, 1990). The effect of these anions in excess should be further studied. Cyanide is also commonly found in electroplating baths but its effect was not tested in the present study because at pH 5.5 HCN gas would be evolved.

5.6 Copper Removal by Immobilized Cells of *Pseudomonas putida* 5-X

5.6.1 Copper Removal Capacity of Immobilized Cells and Breakthrough Curve for Copper Removal

The Cu(II) removal capacity of the immobilized cells of *P. putida* 5-X was only about half of that of the free cells. It was possible that structural changes on the cells were induced by the polymerization of acrylamide as the polymerization reaction used in the present study involved free radical initiation (Sandler and Karo, 1974) and the heat of polymerization did warm up the gel. Moreover, part of cell surface might be shielded by the gel matrix and thus became unavailable to Cu(II) binding.

As shown by the breakthrough curve for Cu(II) removal by immobilized cells of *P. putida* 5-X (Figure 13), the effluent Cu(II) concentration raise rather rapid within the first 50 ml elution volume. This implied that the column of immobilized cells got exhausted of removal capacity quickly and the effluent Cu(II) concentration would reached unacceptable level (the breakpoint) after treating only a small volume of Cu(II)-laden solution. This can be accepted in laboratory scale model studies for elementary investigation of sorption properties. But in practical wastewater treatment this is undesirable. Improvement can be made by

immobilizing more cells and using a more efficient immobilization method which give a higher cell/matrix ratio. The use of linked column should also be considered (Weber, 1985).

It was found that the effluent Cu(II) concentration did not reached the initial concentration even after an elution volume of 300 ml (bed volume of the column was 16 ml). Such phenomenon was also observed in the control experiment although the difference between initial and effluent Cu(II) concentration was smaller. Huang *et al.* (1990) had reported such observation and it was suggested that a slow intracellular uptake process might take place so that a small but constant amount of Cu(II) was removed by the immobilized cells.

Another possible reason was that part of the cell surface capable of binding Cu(II) might have been shielded by the gel matrix after immobilization. Yet the Cu(II) ions might slowly diffuse to these shielded surfaces so that a small amount of Cu(II) ions were still removed over a long period.

However, since the control which contained no cell but only polyacrylamide gel also showed a similar phenomenon, some other mechanism not concerned with cells might also be accountable for such observation. It was possible that over the prolonged perfusion part of the Cu(II) was removed as precipitates by the immobilized cells or gel matrix which effectively acted as a filter. Cu(II) exists principally as Cu^{2+} ions at pH 5.5, but a very small part of it would be in the form of insoluble precipitates (Tien and Huang, 1987). These precipitates might be constantly removed by the filter-like immobilized cells system whenever the Cu(II) containing buffer passed through the column even though the binding sites for Cu(II) ions were saturated.

The use of polyacrylamide gel as matrix for immobilization of cells was a convenient method for laboratory studies. Moreover, unlike the ionotropic gels like alginate and carrageenan, polyacrylamide gel is insensitive to ionic composition in

the environment. Such property is particularly important in treatment of metal-laden wastewater in which ionic composition will be highly variable. However, polyacrylamide will not be a satisfactory matrix for practical use in wastewater treatment. With reference to the desirable characteristics of matrices used for immobilization mentioned previously (section 2.7.1), polyacrylamide gel is weak in mechanical strength, expensive, potentially hazardous to the environment in case of depolymerization (Macaskie *et al.*, 1987b) and of low intrinsic Cu(II) removal capacity. A better method of immobilization must be sought to make the application of the immobilized cells feasible. The knowledge from chemical engineers would be important on this point.

5.6.2 Effect of Retention Time on Copper Removal Capacity of Immobilized cells

As shown by Figure 14, a longer retention time resulted a slightly higher Cu(II) removal capacity of the immobilized cells. Possibly the role of intracellular uptake and diffusion to shielded cell surfaces (as mentioned in section 5.6.1) was more significant when retention time of the Cu(II) solution was long. However, the improvement of removal capacity was not significant practically and the slightly increased removal capacity could not compensate for the largely increased operation time. Moreover, if the increased removal capacity was really caused by increased intracellular uptake, a long retention time would increased the proportion of unrecoverable Cu(II) trapped inside the cells (Huang *et al.*, 1990). Therefore, in other experiments concerning immobilized cells, a retention time of 1.6 minutes was used.

5.6.3 Efficiency of Copper Recovery from Immobilized Cells by Various Eluants

Neither 0.1 N HCl and 0.1 M EDTA solution recovered 100 % Cu(II) from the immobilized cells loaded with the metal. The unrecoverable Cu(II) might be tightly bound to some binding sites, trapped inside the cells or associated with the

cells by chemisorption through chemical reactions with the cellular structures. Other common mineral acids like sulfuric and nitric acid were not used because of their potential oxidizing capacity which may affect the structural integrity of the immobilized cells (Tsezos, 1984).

It was found that the cells exposed to HCl and EDTA were still intact as observed under light microscope. Therefore, both reagents can be used in recovery of Cu(II) and regeneration of the column of immobilized cells. However, HCl was cheaper than EDTA and it would be more economical to use it as a regenerant. In other experiments involving regeneration, 0.1 N HCl was used as regenerant.

5.6.4 Performance of Immobilized Cells on Multiple Loading-elution Cycles

Enhancement of Cu(II) removal capacity of the immobilized cells was observed after the elution with 0.1 N HCl. Similar enhancement effects were observed in the regeneration of Cu(II) loaded *Aspergillus oryzae* biomass by dilute hypochloric acid (Huang *et al.*, 1989) and U(VI) loaded *Penicillium digitatum* biomass by EDTA and carbonate (Galun *et al.*, 1983). The enhanced Cu(II) uptake by *P. putida* 5-X should be due to an increased availability Cu(II) binding sites but not increased intracellular uptake because the second elution recovered over 90 % of Cu(II) removed in the second loading. On the other hand, it was observed that the packed cell volume of *P. putida* 5-X decreased slightly after exposure to 0.1 N HCl. Thus, it was possible that contact with HCl decreased the volume of the bacterial cells so that those cell surfaces shielded by the gel matrix became available for Cu(II) uptake again. Structural changes induced by HCl might also be accountable for such enhancement of removal capacity. The pretreatment of free cells of *P. putida* 5-X by HCl did not show any increased Cu(II) removal capacity (section 4.4) probably due to the lower concentration used (0.01 N).

The recovery of Cu(II) at the five elution cycles was around 90 %. Despite

the test by ANOVA which indicated no significant difference in percentage recovery between elution cycles, both replicates of the experiment showed a tendency of increased percentage recovery after the third elution. For one set of experiment over 100 % of recovery was recorded at the fourth and fifth cycles. It was possible that part of the Cu(II) unrecoverable at the early elutions became recoverable again at the later elution processes.

5.6.5 Treatment of Effluent from an Electroplating Factory by Immobilized Cells

The feasibility of using the immobilized cells for treating effluent from electroplating factory which were heterogeneous in nature was tested in this experiment. The results demonstrated that the immobilized cells may be used in treatment of effluent from electroplating works and the removed Cu(II) could be recovered by over 90 %.

The lowered removal capacity as compared to values obtained in earlier experiments could be due to a couple of reasons. With respect to the metal composition of the sample, it was possible that the excess nickel present might inhibit Cu(II) uptake by immobilized cells, although previous study (section 4.5.4) showed that Ni(II) at equal molarity had no effect on Cu(II) removal capacity of free cells of *P. putida* 5-X. The presence of anions in the effluent sample, whose identity and concentration have not been determined, might also inhibit Cu(II) uptake. Moreover, one should be cautious to compare the present result with those obtained under defined conditions because the initial Cu(II) concentration and pH of the effluent sample, both of them being highly decisive to Cu(II) removal capacity, were different from those of the Cu(II) containing buffer.

6. Conclusion

From the results obtained in the present investigation, it can be concluded that *Pseudomonas putida* 5-X possesses satisfactory properties required by a good sorbent. Its applicability in electroplating effluent treatment seems to depend on the cost of cell mass production and the development of appropriate immobilization method.

Pseudomonas putida 5-X can achieve rapid Cu(II) uptake and possesses satisfactory removal capacity at low Cu(II) concentration to give a residual Cu(II) concentration below 1 mg/l. Only mild interference from metal ions and anions commonly occur in electroplating baths and effluents is found on Cu(II) removal. Cu(II) sorbed can be recovered by about 90 % and removal capacity of the bacterium can be restored after regeneration for at least five loading-elution cycles. All these characteristics are favorable to metal removal and recovery from electroplating effluents.

However, for the biosorbent thus developed to be commercially viable, the cost of culturing the bacterial cells must be minimized because the immobilized cells of *Pseudomonas putida* 5-X is likely to be in competition with many other low cost sorbents derived from agricultural wastes such as xanthates (Wing *et al.*, 1975; Tare and Chaudhari, 1987), straw (Larsen and Schierup, 1981) and pulp (Al-Haj Ali *et al.*, 1987) and certain industrial wastes (Srivastava *et al.*, 1989). Although their heavy metal removal capacities are not as high as that of *Pseudomonas putida* 5-X, their low costs may still justify their application. The use of nutritive waste materials from food industries or any other sources for cell culturing may be a feasible solution and its possibility should be investigated.

Immobilization method is another key point which decides whether the cells of the bacterium can be successfully used as biosorbent for metal removal. As discussed previously, the use of polyacrylamide gel for model study in laboratory

scale is acceptable but not in practical application for wastewater treatment. A cheap, non-toxic matrix which has good mechanical strength and chemical stability and allow high cells/matrix ratio, should be sought to immobilized the cells of *P. putida*. The development of such matrix for immobilization will need extensive research and the knowledge from other disciplines especially chemical engineering would be very important.

7. Summary

7.1 The *Pseudomonas putida* 5-X isolated from a heavy metal polluted nullah was chosen for studies on Cu(II) removal.

7.2 The culture conditions which enhanced Cu(II) removal by *Pseudomonas putida* 5-X were determined as growth in the sulfate-limiting medium at 37 °C for 36 hours.

7.3 The Cu(II) uptake mechanism of *Pseudomonas putida* 5-X was found to be metabolism-independent as presence of glucose and pretreatment of cells with sodium azide did not affected Cu(II) removal. Transmission electron micrograph showed that the Cu(II) accumulated was largely located at the cell surface.

7.4 Pretreatment of cells of *Pseudomonas putida* 5-X by 0.01 N HCl did not affected the Cu(II) removal capacity. Pretreatment by KOH and autoclaving for 10 minutes greatly reduced the removal capacity.

7.5 The cells of *Pseudomonas putida* 5-X removed Cu(II) very rapid and within the first 10 minutes over half of the total removal capacity of the bacterium. A much slower uptake stage was found in the next 90 minutes.

7.6 Cu(II) sorption by *Pseudomonas putida* 5-X at 30 °C over the concentration range 0.5 - 100 mg/l fitted the Freundlich isotherm well.

7.7 Cu(II) uptake by *Pseudomonas putida* 5-X was enhanced at pH higher than 5.5 but inhibited at pHs below this point.

7.8 At equi-molar level Pb(II) ions highly inhibited Cu(II) uptake by *Pseudomonas putida* 5-X. Zn(II) ions inhibited Cu(II) uptake slightly while Ni(II) ions had no effect.

7.9 Borate, chloride and sulfate ions slightly reduced Cu(II) removal capacity of *Pseudomonas putida* 5-X at equi-molar level. Carbonate ions did not affect the removal capacity.

7.10 The Cu(II) removal capacity of the immobilized cells of *Pseudomonas putida* 5-X was only about half of that of the free cells. The removal capacity of the immobilized cells was exhausted rather quickly on the perfusion of the Cu(II) containing buffer as revealed by the breakthrough curve.

7.11 Increased retention time generally led to increased Cu(II) removal capacity of the immobilized cells of *Pseudomonas putida* 5-X. No further enhancement was observed when retention time increased beyond 3 minutes.

7.12 Cu(II) removed by the immobilized cells of *Pseudomonas putida* 5-X was recoverable by 0.1 N HCl and 0.1 M EDTA solution. The recovery efficiency was about 90 % and both eluant performed similarly.

7.13 The immobilized cells of *Pseudomonas putida* 5-X could be used for at least 5 loading-elution cycles. The Cu(II) removal capacity generally increased after the first elution by 0.1 N HCl. The removal capacity of the immobilized cells did not drop below the initial level for at least 5 loading-elution cycles.

7.14 The immobilized cells of *Pseudomonas putida* 5-X could be used to treat an electroplating effluent sample and the Cu(II) removed was recoverable.

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