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STUDY OF MAGNETITE-IMMOBILIZED BACTERIAL CELL SYSTEM FOR REMOVAL AND RECOVERY OF HEAVY METALS FROM ELECTROPLATING EFFLUENT

By

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A Thesis for the Degree of Doctor of Philosophy

Department of Civil and Structural Engineering

The Hong Kong Polytechnic University

August, 2002
Declaration

I hereby declare that this thesis entitled "Study of Magnetite-Immobilized Bacterial Cell System for Removal and Recovery of Heavy Metals from Electroplating Effluent" is original and has not been submitted for other degrees or the like in this University or any other institutes. It does not contain any material, partly or wholly, published or written by others, except those references quoted in the text.

_____________________________
Wang Lei
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Abstract

Cu$^{2+}$ and Ni$^{2+}$ are the major heavy metal ions in electroplating wastewater of Hong Kong. Unlike organic pollutants, which in most cases can eventually be destroyed, heavy metal ions such as Cu$^{2+}$ and Ni$^{2+}$ discharged into the environment tend to persist indefinitely, circularly and eventually throughout the food chain thus causing a series of threats to human. Therefore, the Cu$^{2+}$ and Ni$^{2+}$ must be removed from electroplating effluent before discharging into water bodies. The most common methods for removing heavy metals from wastewater, such as chemical precipitation, ion exchange, electrochemical treatment and evaporative recovery have significant disadvantages, namely, removing metal incompletely, high reagent requirement, generation of toxic sludge or very expensive when the contaminant concentrations are less than 100 mg L$^{-1}$. During the past two decades, biosorption, as an alternative technology, using bacteria, algae and fungi as biosorbent for removing heavy metal has received a great deal of attention. Bacteria are of particular interest because their surface: volume ratio is often relatively high. The present study was thus conducted to investigate the removal and recovery of Cu$^{2+}$ and Ni$^{2+}$ from electroplating wastewater by bacterial cell biomass.

A gram-negative bacterium *Pseudomonas putida* with high Cu$^{2+}$ and Ni$^{2+}$ accumulating capability was isolated from local electroplating effluent. *P. putida* 5-x cells cultured in sulfate limiting medium was found of obviously high Cu$^{2+}$ and Ni$^{2+}$ adsorption capacity compared with those cultured in other media. Higher incubation temperature had a negative effect on Cu$^{2+}$ and Ni$^{2+}$ adsorption of *P. putida* 5-x cell, with effect particularly obvious at the logarithmic growth phase. The bacterial cells harvested in 34-38h and 28-30h had maximum Cu$^{2+}$ and Ni$^{2+}$ adsorption capacity, respectively. Both TEM and X-ray analysis confirmed the above phenomenon. Pretreating the bacterial cells with diluted HCl could
increase their Cu$^{2+}$ adsorption capacity by 24% and Ni$^{2+}$ adsorption capacity by 31%, respectively, mainly due to the degradation of a loose superficial layer outside the fresh cell-capsule.

The adsorption process of Cu$^{2+}$ and Ni$^{2+}$ by fresh cell consisted of two phases, namely a rapid, metabolism-independent metal ions adsorption phase (biosorption) followed by a slow metabolism-dependent bioaccumulation phase, while pretreated cell only consisted of a rapid, metabolism-independent adsorption phase. About 80% of the total Cu$^{2+}$ and Ni$^{2+}$ taken up by the fresh bacterial cells were removed within the rapid adsorption phase. The adsorption capacity of P. putida 5-x cell was obviously affected by changes of pH. The high the pH, the high the Cu$^{2+}$ and Ni$^{2+}$ adsorption capacities. Other cations such as Pb$^{2+}$ and Zn$^{2+}$ might act as competitors of Cu$^{2+}$ and Ni$^{2+}$ to inhibit their removal. The affinity of P. putida 5-x cell biomass to Pb$^{2+}$, Cu$^{2+}$, Zn$^{2+}$ and Ni$^{2+}$ was in order of Pb$^{2+}$ > Cu$^{2+}$ > Zn$^{2+}$ > Ni$^{2+}$. The presence of Cu$^{2+}$ at high concentration inhibited Ni$^{2+}$ adsorption, but when the ratio of mole concentration of Cu$^{2+}$: Ni$^{2+}$ in wastewater reduced to 0.1 below, the inhibition of Cu$^{2+}$ to Ni$^{2+}$ adsorption of P. putida 5-x cell disappeared basically. Anions such as Cl$^-$, SO$_4^{2-}$ and NO$_3^-$ hardly inhibited the Cu$^{2+}$ and Ni$^{2+}$ uptake. The Cu$^{2+}$ and Ni$^{2+}$ binding by P. putida 5-x cell could be considered as adsorption process, their adsorption process obeyed Freundlich isotherms in higher pH solution, while obeyed Langmuir isotherms in lower pH solution. Adsorption isotherms showed that pretreated cell was a better biosorbent either for Cu$^{2+}$ or for Ni$^{2+}$ than fresh cell.

Under suitable conditions, 0.1–0.3 M HCl could effective recover Cu$^{2+}$ and Ni$^{2+}$ from loaded cell biomass with less biomass loss rate. The desorption process was quite rapid, with 95% Cu$^{2+}$ and 99% Ni$^{2+}$ being desorbed in the first 5 minutes. P. putida 5-x cell as biosorbent could be effectively regenerated and reused at least five cycles for removing and recovering Cu$^{2+}$ or
Ni²⁺.

The adsorption capacity of magnetite immobilized *P. putida* 5-x cell to Cu²⁺ and Ni²⁺ appeared to be much higher than using magnetite alone. The Cu²⁺ and Ni²⁺ adsorption by both magnetite alone and magnetite immobilized cells obeyed the Freundlich isotherms of $Q_{M-Cu} = 2.1 C_{e-Cu}^{0.68}$, $Q_{IM-Cu} = 11.9 C_{e-Cu}^{0.74}$ and $Q_{M-Ni} = 0.78 C_{e-Ni}^{0.79}$, $Q_{IM-Ni} = 9.7 C_{e-Ni}^{0.21}$. A two-stage semi-continuous stirred reactor with immobilized *P. putida* 5-x cell as biosorbent was developed to sequentially remove and recover Cu²⁺ and Ni²⁺ from wastewater. In suitable operation conditions, the two-stage system could effectively remove and recover sequentially from synthetic wastewater at least five cycles. Removal efficiency as high as 96% and 97% for Cu²⁺ and Ni²⁺ respectively could be attained, while the concentrations in treated effluents reduced from 30mg/L to around 1 mg/L for Cu²⁺ and to 0.9 mg/L below for Ni²⁺. The Cu²⁺ and Ni²⁺ recovery rate from loaded biosorbent could reach 95% and 98% above, respectively. The treatment efficiency of the two-stage biosorption system to real electroplating wastewater was slightly lower than that to synthetic wastewater due to the interfering of other cations and anions in real wastewater. However, the Cu²⁺ and Ni²⁺ concentration in the treated effluent were also reduced to a concentration that meeting the effluent discharge standard formulated by Environmental Protection Department of Hong Kong, SAR.

The capsules outside the fresh cell of *P. putida* 5-x reduced the adsorption capacity to Cu²⁺ due to the Cu²⁺-bridging formed by binding divalent Cu²⁺ on electronegative groups in the capsule, which might induce a conformation change within the capsule, and thus resulted in some metal-binding sites on the cell outer membrane or PEG layer becoming inaccessible for Cu²⁺ binding. Chemical and physical treatment during the separation course of cell envelopes would liberate more metal-binding sites on cell envelope which were heavy.
metal accessible. Therefore the separated cell envelope increased 4 times more Cu$^{2+}$ adsorption capacity than that of fresh intact cell. Separated PEG layer, outer membrane and inner membrane all played roles on Cu$^{2+}$ adsorption by cell envelope of *P. putida* 5-x. The total contribution of PEG layer, outer membrane and inner membrane to Cu$^{2+}$ adsorption of the cell envelope was in order of outer membrane > inner membrane > PEG layer materials. The variation of Cu$^{2+}$ adsorption capacity of cell envelope in different cell growth phases was due to the variation of PEG layer content, outer and inner membrane content in cell envelope, and the variation of Cu$^{2+}$ adsorption capacity of outer membrane and inner membrane in different cell growth phases. The variation of both phospholipids and lipopolysaccharides content in outer membrane was found attributed to the variation of Cu$^{2+}$ adsorption capacity of the outer membrane in different cell growth phases, nevertheless, the variation of only phospholipids content in inner membrane might result in the variation of its Cu$^{2+}$ adsorption capacity in different cell growth phases.
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Chapter 1: Introduction

1.1 Heavy Metal Pollution in Environment

Heavy metals, such as copper (Cu), zinc (Zn), nickel (Ni), cobalt (Co), silver (Ag), cadmium (Cd), and chromium (Cr), are commonly found in municipal sewage and industrial effluent. These common heavy metals are extensively used in the electroplating and metal-processing industries. Barium (Ba), lead (Pb), iron (Fe) and mercury (Hg), are the major waste constituents in the manufacturing of paints, paper, plastics, batteries, alloys, refractoriness, and scientific instruments [Lester et al., 1979; Sawyer and McCarty, 1978].

In Hong Kong and the South China, copper and nickle pollution arises mainly from the effluents discharged from the electroplating and printed circuit board factories [Environment Protection Department, 1997]. Copper of 20~30 mg L\(^{-1}\) and Nickle of 30~40 mg L\(^{-1}\) were commonly found in the electroplating effluent of Hong Kong [Chiu, 1987]. It was reported that for a typical printed circuit board factory, about 55 kg of copper were discharged per month into Hong Kong waters before the enactment of the Water Control Ordinance [Wong MF, 2000]. High levels of copper, nickle have also been found in the sediment of Victoria Harbour.

For nearly a century, industrial discharges of heavy metal-laden wastewater in relation to the environment and public health have been of concerned. Numerous investigations on effect of heavy metal on environment and human have then been carried out. Unlike organic pollutants, which in most cases can eventually be destroyed, heavy metal discharged into environment tend to persist indefinitely, circularly and eventually throughout the food chain thus causing a series of threats to human and organism [Chua
and Hua, 1996; Cooke et al., 1990; Deniseger et al., 1990; Sag et al, 1995b]. Thus the presence of heavy metal ions in water possess serious environmental and human health hazards because of its toxicity, its tendency to bioaccumulate, and its abundance and persistence in the environment [Kratochvil and Volesky, 1998]. The toxicity of Ni\(^{2+}\), Cu\(^{2+}\) to human and fish has also been well documented [Taylor et al., 1979; Nriagu, 1980; Flemming and Trevors, 1989; Codina et al., 1994].
1.2 Current Treatment Technology for Heavy Metal Removal

From 1986, the Hong Kong government has implemented the Water Pollution Control Ordinance in which metal-processing industries were required to pre-treat or detoxify the metal-rich effluents before discharging them into the nearby aqueous bodies.

The most commonly used technologies for removing heavy metals from wastewater include chemical precipitation, ion exchange, electrochemical treatment and evaporative recovery. However, these procedures have significant disadvantages, namely, removing metal incompletely, high reagent requirement, generation of toxic sludge or very expensive when the contaminant concentrations are less than 100 mg L⁻¹ [Sag et al., 1995b]. Otherwise, additional chemical dosage may impact the microorganism activity of subsequent activated sludge process for removal of COD.
1.3 Biosorption for Removal of Heavy Metal

The use of biological materials as biosorbent for heavy metal adsorption and recovery has received a great deal of attention in the past two decades due to their good performance and low cost [Volesky, 1994; Kratochvil and Volesky, 1998]. Microorganisms including bacteria, microalgae, fungi and yeast could adsorb or accumulate large amount of heavy metals even when they are dead, bacteria are of particular interest because of their high cell surface area per volume, [Bakkaloglu, 1998; Gutnick et al., 2000; Kratochvil, 1998; Leung et al., 2000; Lo et al., 1999; Mameri, 1999; Volesk, 1994; Wong et al, 1993a,b. Yetis, 1998].

The accumulation of metals by biological material, such as bacteria, microalgae and fungi or their separated cell components, without metabolism-dependent is known as biosorption, which can be considered a collective term for a number of passive accumulation processes including ion exchange, coordination, complexation, adsorption and microprecipitation [Volesky and Holan, 1995]. The biological materials are capable of removing even trace levels of metal ions. They can also be used to recover rare, precious or strategic metals from waste solutions. The abundant bacterial biomass can be obtained inexpensively, as they are waste byproducts of large-scale industrial processes such as fermentation and activated sludge wastewater treatment. Hence, biosorption coupled with desorption may provide an economic and effective alternative for removal and recovery of heavy metal.

Electroplating wastewater appears of high toxicity to most microorganism due to the presence of heavy metal such as copper, nickel and chromium. However, certain bacteria could still be found in electroplating wastewater. These bacteria indigenous in electroplating wastewater may possess capability of detoxifying or resisting high
concentration heavy metal in solution. It had been confirmed use of bacterial cell isolated from electroplating effluent with high heavy metal adsorption capacity as biosorbent that for removing heavy metal was more efficient [Wong et al 1993b]. This motivated us to evaluate the feasibility and ability of using microorganisms indigenous in electroplating wastewater to remove copper and nickel from electroplating wastewater.
1.4 Objective and Study Scopes of the Project

It is of importance to isolate more microbial strains that could uptake metals with high adsorption capacity and also to design better bio-process that could effectively remove and recover heavy metals from aquatic systems. For optimizing design and operation of biosorbent system for metals removal and recovery from electroplating effluent, a thorough understanding of biosorption characteristics and mechanism of bacterial cell biomass with high heavy metal binding capacity isolated from electroplating effluent is needed.

From the practical operation point of view, biosorbent regeneration, transport and disposal contributed to major cost compared with biosorbent production [Atkinson et al., 1998]. In order for biosorption to become competitive with existing technology, metal adsorption capacity of biomass should be improved as far as possible to reduce the using amount of biosorbent and thus minimizing the sludge regeneration, disposal and transport cost. For improving adsorption capacity of biosorben for heavy metal, the screening technology for bacteria with high Cu²⁺ and Ni²⁺ adsorption capacity, the optimization of biosorbent preparation techniques, and the optimal adsorption conditions and process design should be studied.

In addition, a clear understanding of the biosorption mechanisms, such as adsorption site and active groups adsorption related, and contribution of cell surface components and chemical ingredients to heavy metal adsorption, and the adsorption isotherms, are very crucial for the development of microbial adsorption process. These results can serve as a useful aid for enhancing metal adsorption capacity of bacterial cells through modifying cell surface structure and components using either DNA recombination technology or metabolic regulation techniques.
The overall objective of this research project was to isolate a bacterial strain which is of high adsorption capacity to both Cu\(^{2+}\) and Ni\(^{2+}\) from electroplating effluent as biosorbent, and then develop a novel and economical biosorption process for removing and recovering Cu\(^{2+}\) and Ni\(^{2+}\) from electroplating effluent using the bacteria-based biosorbents.

Concretely, in the present study, the extensively screening of bacteria with high Cu\(^{2+}\) and Ni\(^{2+}\) adsorption capacity from electroplating effluent was carried out using a novel plate colour developing technology. The Cu\(^{2+}\) and Ni\(^{2+}\) adsorption capacities of the isolated cell biomass were enhanced through optimization of cell preparation conditions and application of cell pretreatment techniques. Factors affecting biosorption of cell biomass have been systematically and extensively studied, namely, the effects of cell culture condition, cell age, biomass pretreatment, solution pH, competing cations and anions and adsorption temperature on adsorption. The reusability of the biomass was studied by repeating biosorption and desorption operations in order to further evaluating the feasibility of applying isolated cell biomass to practical heavy metal removal process. In order for biosorption to function efficiency in rigorous industrial application, and for the purpose of biosorbent reuse and easy separation from liquid phase, immobilized cell biomass was studied and evaluated.

Finally a laboratory scale bio-reactor based on magnetite-immobilized cells would be developed to evaluate the feasibility of the immobilized cell as biosorbent for Cu\(^{2+}\) and Ni\(^{2+}\) removal from electroplating effluent. Feasibility of subsequent Cu\(^{2+}\) and Ni\(^{2+}\) recovery and biosorbent regeneration would also investigated.

In addition, a clear understanding of the biosorption mechanisms, such as adsorption sites and active groups on the cell surface, contribution of cell surface components and chemical ingredients to heavy metal adsorption, biosorption kinetics, the uptake capacity
and affinity of bacterial cell biomass to Cu\(^{2+}\) and Ni\(^{2+}\), and the adsorption isotherm is very crucial for developing, improving and controlling microbial adsorption processes. Thus, an extensive study on the biosorption mechanisms was also investigated by using techniques of biochemical separation, scanning electron microscopy (SEM) and transmission electron microscopy (TEM), X-ray energy dispersion analysis, (EDAX).
Chapter 2: Literature Review

This literature review is organised into four sections:

1. The sources of heavy metals and their pollution problems in environment

2. Chemistry and toxicity of heavy metal to environment and human

3. Conventional treatment methods for heavy metal removal from environment

4. Application and progress of biosorption on heavy metal removal

Heavy metals have been defined in many ways [Nieboer and Richardson, 1980; Phipps, 1981]. In general, chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), copper (Cu), zinc (Zn), molybdenum (Mo), silver (Ag), mercury (Hg), cadmium (Cd), nickel (Ni) and metals with atomic mass larger than that of sodium are widely accepted as heavy metals. Besides, metals from groups IIA, IIB, IVB and VB of the element periodic table, namely aluminum (Al), beryllium (Be), tin (Sn), thallium (Th), lead (Pb) and bismuth (Bi) as well as the metalloids arsenic (As), selenium (Se) and antimony (Sb) are often included toxic heavy metals.

Most commonly, heavy metals are 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].

2.1 Heavy Metal Pollution in Environment
Metals occur naturally, but large-scale release to the aquatic environment requires human intervention. 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. Industrial wastewater, such as electroplating wastewater, metal-processing wastewater, is another obvious source of metal discharge. In addition there are the more diffuse sources of urban runoff and leachate from solid waste disposal sites, both of which may be rich in metals. The metals released may be airborne, contained in water runoff or flow, or dumped as solid residues [Patterson, 1987; Beijer and Jemekyll, 1986].

Heavy metals, such as copper (Cu), zinc (Zn), nickel (Ni), cobalt (Co), silver (Ag), cadmium (Cd), and chromium (Cr), are commonly found in municipal sewage and industrial effluent. These common heavy metals are extensively used in the electroplating and metal-processing industries. Barium (Ba), lead (Pb), iron (Fe) and mercury (Hg), are the major waste constituents in the manufacturing of paints, paper, plastics, batteries, alloys, refractories, and scientific instruments [Lester et al., 1979; Sawyer and McCarty, 1978].

The Environmental Protection Department of Hong Kong reported that more than two million tonnes of sewage and industrial wastewater were produced every day in Hong Kong. Two tonnes of heavy metals were discharged daily into Hong Kong waters [Environmental Protection Department, 1997a].

In Hong Kong, the major contribution of heavy metals is from the industrial discharge of effluent from metal finishing and electroplating industries [Environmental Protection Department, 1989]. Electronics and printed circuit board industries are other local sources of heavy metal pollutants. The industries in Southern China that produce metal-laden wastewater are more diversified, and may also indirectly affect the Hong Kong
environment. Batteries and plastics industries produce cadmium wastewater. Chromium is commonly used in the manufacture of alloys, refractories, chemical catalysts and other chromic salts. Mercury is used in the production of amalgams, scientific instruments, batteries, arc lamps and gold and silver mining. Nickel, copper, zinc, and cobalt are common metals for the electroplating processes. Zinc is also widely used for making galvanised pipes. Although lead is seldom used because of its toxicity, it is widely used in the past in the production of lead service pipes, lead based paints and catalytic tetraethyl-lead gasoline for internal-combustion engines.

It has been shown by the Hong Kong Environment Protection Department [Environment Protection Department, 1997b; 1998; 1999] that the most serious heavy metal contents were at Kau Wa Keng Stream (Cd), Kai Tak Nullah (Cr), Yuen Long (Cu and Pb), and River Indus (Zn). A summary of the highest heavy metal contents measured between 1996 and 1998 is tabulated in Table 2.1
Table 2.1

A summary of heavy metals in various river water quality monitoring stations. (Data presented are in annual medians of monthly samples; figures in brackets are annual ranges) [Environment Protection Department, 1997b; 1998; 1999]

(a) Concentration of cadmium

<table>
<thead>
<tr>
<th>Kau Wa Keng Stream - KW 3</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>1.20</td>
<td>1.75</td>
</tr>
<tr>
<td>(μg L⁻¹)</td>
<td>(0.10 - 2.40)</td>
<td>(0.70 - 3.70)</td>
</tr>
</tbody>
</table>

(b) Concentration of chromium

<table>
<thead>
<tr>
<th>Kal Tak Nullah - KN4</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr</td>
<td>100</td>
<td>14.5</td>
</tr>
<tr>
<td>(μg L⁻¹)</td>
<td>(1.0 - 610.0)</td>
<td>(1.0 - 100.0)</td>
</tr>
</tbody>
</table>
(c) Concentrations of copper and lead

<table>
<thead>
<tr>
<th></th>
<th>Yuen Long - YLI</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu (μg L⁻¹)</td>
<td>156.5</td>
<td>40.5</td>
<td>24.5</td>
</tr>
<tr>
<td>Pb (μg L⁻¹)</td>
<td>13.0 (2.0 - 24.0)</td>
<td>4.0 (2.0 - 19.0)</td>
<td>2.0 (1.0 - 18.0)</td>
</tr>
</tbody>
</table>

(d) Concentration of zinc

<table>
<thead>
<tr>
<th></th>
<th>Piver Indus - IN2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn (μg L⁻¹)</td>
<td>345 (120.0 - 6,800.0)</td>
<td>380 (110.0 - 4,400.0)</td>
<td>855 (100.0 - 11,000.0)</td>
</tr>
</tbody>
</table>
In the case of marine water bodies, on the other hand, high concentrations of heavy metals in coastal waters [Chan et al., 1974], rivers entering into estuaries [Wong et al 1980] and tissues of coastal marine organisms [Chan et al., 1990; Chu et al 1990; Phillips, 1989] have been reported. Although concentrations of toxic metals in sea-water are usually below the concentrations which directly cause mortality, several investigations [Sullivan et al., 1983; Lang et al., 1980; Mirkes et al., 1978] reported that sub-lethal concentrations may still affect the physiological and behavioural functioning of the organisms. Lo and Fung (1992) conducted a study on the pollution of heavy metals in the sediments at Hebe Haven of Sai Kung, Hong Kong. They found concentrations as high as 0.93, 120, 131, 42.3 and 50.9 mg kg\(^{-1}\) for cadmium, chromium, zinc, copper and lead, respectively. Recently, Owen and Sandhu (2000) reported that chromium levels ranged between 14-30 mg kg\(^{-1}\) and Nickel ranged between 4-10 mg kg\(^{-1}\) at the Inner Tolo Harbour, but rose to 17 mg kg\(^{-1}\) at Tolo channel. Very low concentration of cadmium was reported.

Additionally, Lam et al. (1997) reported their study on the pollution of chromium, copper and nickel in coastal estuarine sediments by domestic and industrial effluents. In the overall metal content in all labile geo-chemical phases, the levels of chromium, copper and nickel in sediments of Victoria Harbour were significantly higher than those of Tolo Harbour. Environment Protection Department (1997b) showed that the sediments in Victoria Harbour and Tsuen Wan contained relatively high concentrations of copper (> 65 mg/kg) and mercury (> 75 mg kg\(^{-1}\)). Wong et al., (1995) reported their measurements of heavy metals in Victoria Harbour. The ranges of heavy metal concentrations measured were 98-259 mg kg\(^{-1}\) for zinc, 28900-34100 mg kg\(^{-1}\) for iron, 47-71 mg kg\(^{-1}\) for lead, 2.6-3.3 mg kg\(^{-1}\) for cadmium, 58-171 mg kg\(^{-1}\) for chromium, 45-922 mg kg\(^{-1}\) for copper and 24-64 mg kg\(^{-1}\) for nickel. Bottom sediments in typhoon shelters were particularly heavily
polluted with, for example, copper levels from Kowloon Bay reaching 6250 mg kg$^{-1}$ in 1994 [Environmental Protection Department, 1995]

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 compositions of effluents from sixteen local electroplating factories have been investigated [Chiu et al., 1987]. As shown in Table 2.2, chromium, copper, nickel and zinc occurred at electroplating effluent, particular of copper and nickel at high concentration of 20–30 mg L$^{-1}$ and 30–40 mg L$^{-1}$, respectively. Additionally, it has been reported that for a typical printed circuit board factory, about 55 kg of copper were discharged daily into Hong Kong waters before the enactment of the Water Control Ordinance [Wong MF, 2000].

Copper, nickel and some other heavy metal ions were listed as grey or black metals by European Union (Table 2.3) [Wase and Forster, 1997]. Black metals mean which are the most toxic and must be controlled drastically and removed from circumstance. Grey metals mean which should be controlled severely and reduced to trace concentration. Copper and nickel belong to the grey metals.
Table 2.2: The main composition of the effluent collected from an electroplating factory

<table>
<thead>
<tr>
<th>Metal</th>
<th>Concentration (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>16-18</td>
</tr>
<tr>
<td>Cr</td>
<td>0.1-0.3</td>
</tr>
<tr>
<td>Ni</td>
<td>31-36</td>
</tr>
<tr>
<td>Zn</td>
<td>1-1.5</td>
</tr>
<tr>
<td>Pb</td>
<td>trace</td>
</tr>
<tr>
<td>Suspended solids</td>
<td>20-50</td>
</tr>
</tbody>
</table>

Table 2.3 Black and Grey List metals by EU

<table>
<thead>
<tr>
<th>Black List</th>
<th>Grey List</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium</td>
<td>Chromium</td>
</tr>
<tr>
<td>Mercury</td>
<td>Copper</td>
</tr>
<tr>
<td></td>
<td>Lead</td>
</tr>
<tr>
<td></td>
<td>Nickel</td>
</tr>
<tr>
<td></td>
<td>Zinc</td>
</tr>
</tbody>
</table>
The Water Pollution Control Ordinance of Hong Kong has been enacted since 1986 to control water pollution. The metal-rich effluents before discharged into the aqueous environment should be pre-treated or detoxified. In addition, for efficient removal of organic pollutant, the maximum allowable heavy metal concentration in waste effluent discharged into sewage treatment plants with microbial treatment is 1 mg L⁻¹ Cu²⁺ and 2 mg L⁻¹ Ni²⁺ with flow rate between 400 and 600 m³/day [Environmental Protection Department, 1991].
2.2 Characteristic and Toxicity of Heavy Metal in the Environment

Ingestion of heavy metals is harmful to human being and some biota. For instance, methyl-mercury is produced from inorganic mercury as a consequence of microbial activity. Its compounds accumulated in fish tissues and transferred to brain tissues of human when consumed. Such compounds accumulated and stored in brain tissues have been linked to mental impairment and in some cases it has caused death. The well-known outbreak of methyl-mercury poisoning in Japan following the consumption of polluted fish and shellfish can be referred to study group on Minamata Disease [Tsuchiya, 1969].

For another example, as high as 3.4 mg L⁻¹ of arsenic in well water in the Cordoba region of Argentina was measured. Such high level of arsenic has been recorded to cause 165 deaths. A high proportion of the deaths was due to cancer of the respiratory system and gastrointestinal tract [Pershagen, 1983]. The toxicity of arsenic is dependent on its oxidation state, chemical form and solubility in the biological media [Subramanian, 1988].

In general, the toxicity of the metals depends on the physico-chemical properties of metals and their position in the periodic chart form. The LD₅₀ test is one of the criteria for assessing metal toxicity. Expressing LD₅₀ on a molar basis is a good index for comparing the toxicity of similar metals. Other terms such as MDNF (maximum dose never fatal in 24 hrs) and MDAF (minimum dose always fatal) are useful in expressing lethal doses. The ratio between the essential and toxic dose will be the best way of expressing the toxicity of essential metals, such as Se, which prove toxic at higher concentration levels.

Electro-positivity of metals and increased solubility of metal salts increase the toxicity. Solubility enhances both the gastrointestinal absorption of orally ingested compounds and the distribution of these. Metals can bind with active ligand groups of free or
membranous biological macromolecules, structural tissues, and cellular constituents. Metal binding with organic OH, PO₄, and COOH groups is of the strong electrovalent type. The strong binding ability between metal and organism structure and tissue result in the metal toxicity to organism, the strength of the bonding influences the metal toxicity in biological systems. Enzymatic activities may be inhibited by the binding of heavy metal ions to enzyme molecules, thus masking the catalytic sites or altering the conformations of the biomolecules, especially enzymes whose activities depend on sulphhydryl and amino groups, as heavy metals have high affinity for ligands containing nitrogen and sulfur donors [Flemming and Trevors, 1989]. Besides, nucleic acids and metabolises may also be targets of heavy metal toxic actions [Tyler et al., 1989].

The copper and nickel are the major heavy metal in electroplating effluent of Hong Kong. Unlike organic pollutants, which in most cases can eventually be destroyed, these heavy metals without treatment discharged into near aquatic environment tend to persist indefinitely. They circulate and eventually accumulate throughout the food chain, thus possessing a series of threats to animals and man. The toxicity of copper and nickel to mammal and aquatic organism was discussed in detail as belows:

Copper is a transition metal with 3 common oxidation states: Cu⁺ (metal), Cu²⁺ (cuprous ion) and Cu³⁺ (cupric ion). Cupric ion (Cu²⁺) 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. Cu²⁺ starts to precipitate above pH 5.5. But in the presence of organic or inorganic ligands, Cu²⁺ get complexed and remains free in solution. It can be strongly complexed by electron donor groups (P-, O-, N- and S-containing groups) in organic compounds [Flemming and Trevors, 1989].

Nickel forms stable di- and trivalent salts. Nickel coordination complexes involve coordination numbers 4 and 6. Divalent ion is the most commonly occurring species in
water. Many studies revealed that copper and nickle appear toxicity to aquatic life, mammals, and microorganism.

2.2.1 Toxicity of Copper and Nickle to Mammals

The metabolism of copper has been well studied [Peisack et al., 1966; Van Campen, 1971]. Copper, exhibiting uni- and divalent is absorbed from both stomach and intestine, most readily in the duodenal region. Copper is an essential metal involved in a number of enzymes; e.g., phenol oxidases and cytochrome oxidases.

Excessive intake of copper results in its accumulation in the liver. High dietary copper intakes of 500 ppm, about 100 times the usual intake, are tolerated by rats despite a 14-fold increase in liver copper. Beyond this quantity erythrocytes cannot sequester all the copper and the copper is suddenly released into the blood serum resulting in hemolysis. Pigs, fed dietary copper at 600 to 750 ppm show high serum aspartate, amino transferase and ornithine carbamyl transferase activities; these are indicative of general tissue damage. Copper toxicity is increased by low Mo, Zn and SO₄²⁻ intake. In general, 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. The toxicity of some copper salts is summarised in Table 2.4
Table 2.4 Toxicity of some copper salts to mammals [Luckey, 1975]

<table>
<thead>
<tr>
<th>Compound</th>
<th>Animal</th>
<th>Route</th>
<th>Toxicity Test</th>
<th>Dosage expressed as mg of dose</th>
<th>compound/Kg</th>
<th>metal/Kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper carbonate</td>
<td>rat</td>
<td>oral</td>
<td>LD₅₀</td>
<td>159</td>
<td>81.7</td>
<td></td>
</tr>
<tr>
<td>Copper chloride</td>
<td>rat</td>
<td>oral</td>
<td>LD₅₀</td>
<td>140</td>
<td>66.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>guinea pig</td>
<td>sc</td>
<td>LD₅₀</td>
<td>100</td>
<td>47.2</td>
<td></td>
</tr>
<tr>
<td>Copper nitrate</td>
<td>rat</td>
<td>oral</td>
<td>LD₅₀</td>
<td>940</td>
<td>318</td>
<td></td>
</tr>
<tr>
<td>Copper sulfate</td>
<td>mouse</td>
<td>iv</td>
<td>LD₅₀</td>
<td>50</td>
<td>12.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>oral</td>
<td>LD₅₀</td>
<td>300</td>
<td>76.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>guinea pig</td>
<td>iv</td>
<td>LD₅₀</td>
<td>2</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rabbit</td>
<td>iv</td>
<td>LD₅₀</td>
<td>4.5</td>
<td>1.15</td>
<td></td>
</tr>
</tbody>
</table>
Nickel is an essential trace metal for mammals according to Nielsen (1971). Ni forms stable di- and trivalent salts. Ni coordination complexes involve coordination numbers 4 and 6. Involvement of nickel in enzyme activation, hormonal action, structural stability of biological macromolecules and general metabolism has been extensively reviewed [Browning, 1969; Underwood, 1971].

Metallic nickel and its soluble salts in large doses are toxic to animals. The lethal doses are listed in Table 2.5. Parenteral administration and chronic inhalation of nickel dust produce tumors [Hueper, 1958]. Ni which can bind to nucleic acids thus can inhibit unwinding and rewinding of DNA in vitro tend to be carcinogenic. Ni from tobacco smoke is reported to be involved as a pulmonary carcinogen [Sunderman and Sunderman Jr., 1961].

Ingestion of nickel at 250, 500, and 1000 ppm does not affect the growth rate or reproduction in mice, rats, chicken and monkeys [Phatak and Patwarinhan, 1950, 1952]. More than 1000 ppm dietary Ni in mice elicited growth retardation; reduction in the activity of cytochrome oxidase and isocitrate dehydrogenase of liver, malic dehydrogenase of kidney, and cytochrome oxidase and malic dehydrogenase of heart is also observed. Survival of offspring of mice fed 1600 ppm of Ni is reduced [Weber and Reid, 1969].
<table>
<thead>
<tr>
<th>Compound</th>
<th>Animal</th>
<th>Route</th>
<th>Toxicity</th>
<th>Dosage expressed as mg of dose compound/Kg</th>
<th>metal/Kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nickel (colloidal)</td>
<td>dog</td>
<td>iv</td>
<td>LD</td>
<td>--</td>
<td>10-20</td>
</tr>
<tr>
<td>Nickel oxide</td>
<td>cat</td>
<td>iv</td>
<td>LD</td>
<td>12.7</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>dog</td>
<td>iv</td>
<td>LD</td>
<td>9.0</td>
<td>7</td>
</tr>
<tr>
<td>Nickel chloride</td>
<td>dog</td>
<td>iv</td>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>40-80</td>
<td>18-36</td>
</tr>
<tr>
<td>Nickel sulfate</td>
<td>guinea pig</td>
<td>sc</td>
<td>LD</td>
<td>62</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>rabbit</td>
<td>sc</td>
<td>LD</td>
<td>500</td>
<td>111.6</td>
</tr>
<tr>
<td></td>
<td>rabbit</td>
<td>iv</td>
<td>MLD</td>
<td>35.8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>cat</td>
<td>iv</td>
<td>LD</td>
<td>71.6</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>dog and cat</td>
<td>sc</td>
<td>LD</td>
<td>500</td>
<td>111.6</td>
</tr>
<tr>
<td></td>
<td>dog and cat</td>
<td>iv</td>
<td>MLD</td>
<td>89.5</td>
<td>20</td>
</tr>
</tbody>
</table>
2.2.2 Toxicity of Copper and Nickel to Other Aquatic Organism in Environment

Many studies had agreed that 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].

There is sufficient information for salmonid species to demonstrate that the adverse effect concentrations decrease with increasing duration of exposure to copper [Mance, 1987 p76]. There are few data relevant to the toxicity of copper to different life stages of the same species. Fish on a low carbohydrate diet had a 6-day LC$_{50}$ of 0.408 mg L$^{-1}$ compared with a 6-day LC$_{50}$ of 0.246 mg L$^{-1}$ for fish on a high carbohydrate diet. The other diets produced an intermediate response.

Exposure of *Pimephales promelas* to cupric acetate resulted in a 4-day LC$_{50}$ (0.66 mg L$^{-1}$) which was considerably greater than is typical for the CuSO$_4$ tested under similar conditions (0.112 to 0.25 mg L$^{-1}$). This may indicate that organic forms of copper are less toxic, [Alabaster and Lloyd, 1980; USEPA, 1980; Spear and Pierce, 1979].

Mance (1987, p77) indicated that for the salmonid species at least, increased temperature has the effect of decreasing the toxicity of copper. The similarity of response in relation to water hardness between salmonid and cyprinid species is not evident for temperature and no relationship can be determined for the two non-salmonid families.

The lowest adverse effect concentration was 0.002 mg L$^{-1}$ which reduced the ability of *Salmo gairdneri* to resist bacterial infection [Knittel, 1980]. However, in a separate study, exposure to 0.01 and 0.22 mg litre$^{-1}$ had no such effect, [Snarski, 1982].

The toxicity of copper to marine life was well-documented compared with the
information for the other heavy metals. However, there is still a lack of long-term studies with only two extending beyond 28 days. Comparison of the 4-day LC$_{50s}$ between phyla suggests that all are similar, apart from the annelids which appear more sensitive to copper. Acute studies of the fish Trachinotus carolinus and the annelid Nereis diversicolor indicated that low salinity increases the toxicity of copper [Mance, 1987].

For fish the response of young life stages is such that the 4-day LC$_{50s}$ for embryos (0.028 mg L$^{-1}$) and larvae (0.136 mg L$^{-1}$) are less than the majority of results for adult fish (0.129 to 0.510 mg L$^{-1}$).

The most sensitive species tested is the hydrozoan Campanularia lexuosa for which colony growth was stimulated by copper concentrations of 0.001 and 0.01 mg L$^{-1}$ but reduced by concentrations of 0.015 and 0.025 mg L$^{-1}$ after 16 days' exposure. An exposure of 8 days at 0.05 mg L$^{-1}$ halted growth of the colonies completely, [Stebbing, 1981]

The toxicity of nickel to freshwater fish has been extensively reviewed [Taylor et al, 1979; Nriagu, 1980; USEPA 1980; Mance and Yates, 1984], and the main conclusions have been supported by the information in Table 2.6. In short-term exposures both salmonid and non-salmonid species are equally sensitive to nickel, especially in soft water. Unfortunately there are no data for long exposures of non-salmonid species. Comparison of the 4-day LC$_{50s}$ demonstrates the significance of water hardness in reducing nickel toxicity, an effect which is more pronounced for non-salmonid species. This may lead to an apparent difference in the relative sensitivity of the two groups of species between soft and hard waters. With increased duration of exposure, the adverse effect concentrations was found to decline and appeared to be stabilised with an approximate minimum of 0.5mg L$^{-1}$. 

25
Table 2.6 Toxicity of nickel salts to freshwater fish [Mance, 1987]

<table>
<thead>
<tr>
<th>Species</th>
<th>Life stage</th>
<th>Hardness (mg L⁻¹)</th>
<th>Chemical</th>
<th>Concentration (mg L⁻¹)</th>
<th>Duration (days)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmo gairdneri</em></td>
<td>2-month</td>
<td>82-132</td>
<td>Ni(NO₃)₂</td>
<td>35.5</td>
<td>4</td>
<td>LC₅₀</td>
</tr>
<tr>
<td></td>
<td>1-year</td>
<td>240</td>
<td>NiSO₄</td>
<td>32</td>
<td>2</td>
<td>LC₅₀</td>
</tr>
<tr>
<td></td>
<td>Juvenile</td>
<td>250</td>
<td></td>
<td>1.2</td>
<td>70</td>
<td>LC₅₀</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>10</td>
<td></td>
<td>7</td>
<td>4</td>
<td>LC₅₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td></td>
<td>25</td>
<td>4</td>
<td>LC₅₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td></td>
<td>50</td>
<td>4</td>
<td>LC₅₀</td>
</tr>
<tr>
<td><em>Salmo salar</em></td>
<td>8-month</td>
<td>11</td>
<td>Ni(NO₃)₂</td>
<td>25</td>
<td>4</td>
<td>LC₅₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.4</td>
<td>40</td>
<td>LC₅₀</td>
</tr>
<tr>
<td><em>Anguilla rostrata</em></td>
<td>adult</td>
<td>53</td>
<td>NiSO₄</td>
<td>14</td>
<td>1</td>
<td>LC₅₀</td>
</tr>
<tr>
<td><em>Cyprinus carpio</em></td>
<td>eggs</td>
<td>128</td>
<td></td>
<td>6</td>
<td>3</td>
<td>52% hatchin</td>
</tr>
<tr>
<td></td>
<td>Larvae</td>
<td>128</td>
<td></td>
<td>8.5</td>
<td>3</td>
<td>LC₅₀</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>53</td>
<td></td>
<td>38.2</td>
<td>1</td>
<td>LC₅₀</td>
</tr>
</tbody>
</table>
Nickel was believed to be relatively low-toxic to marine organisms. However, marine planktonic crustaceans, bivalve larvae and embryonic echinoderms are more sensitive [Mance 1987 p180]. In the latter organisms, abnormal development results from exposures of 20 h to concentrations between 0.05 and 0.58 mg L$^{-1}$ [Rehwoldt et al, 1971], for adult *Asterias forbesi* is 150 mg L$^{-1}$ [Eisler and Henneky, 1977]. That means that for short-term tests adult fish are less sensitive than the invertebrates tested. But due to the complete absence of long-term studies of the toxicity of nickel to either fish or invertebrates, the chronic toxicity of nickel to marine life is not affirmatory.

### 2.2.3 Bioaccumulation and Biomagnification of Heavy Metal in Biota

In view of acute toxicity point, the toxicity of copper and nickle to mammals and aquatic organism is relative low, but unlike organic pollutants, which in most cases can eventually be destroyed, these heavy metal released into environment tend to persist indefinitely. They circulate and eventually accumulate in tissue throughout the food chain, thus possessing a series of chronic threats to animals and man. Thus apart from the direct effects of metals on aquatic organisms resulting from the concentrations in water, the effect of heavy metal bioaccumulation in tissue along the food chain also should be considered.

**Bioaccumulation of metal in aquatic organism:**

Metal added to either fresh or tidal waters tends to be removed by adsorption onto particles or by chemical transformation into an insoluble form. Thus sediment concentrations are normally higher than those in the overlying water. Shellfish, bivalves and some aquatic biota, have higher metal concentration in tissues than in water. At the
primary production level, macrophytes rooted in these enriched sediments tend to have greater concentrations than the sediment. This is because there is uptake not just from the sediment but also from the water itself. This is also true for the algae, whether attached or planktonic, which contain higher concentrations than the surrounding water.

The concentrations of metals in aquatic organisms vary because they reflect the net effect of two competing processes, that of uptake and of depuration. The balance between the two will depend upon the ambient water concentrations and the relative rates of the two processes. It is also affected by a number of factors that may introduce a seasonal pattern of variation.

The ambient concentration of metal in the water will itself vary because of changes in the available dilution, in the rate of dispersion and in the rate of addition of metal. Apart from these variations in the availability of the metal to the organism other factors, such as physiological condition, growth age, salinity and temperature and pollutant interactions [Love, 1970; Phillips 1976 and 1980, Pentreath, 1976; Rolley et al. 1984; Grimshaw et al, 1976], also affect the rate of accumulation of metals.

**Biomagnification of metal**

For the organic-chlorine pesticides a biomagnification along the food chain has been demonstrated, with progressively greater concentrations in herbivores, primary carnivores and then secondary or top carnivores [Phillips, 1980]. This phenomenon of biomagnification is distinct from bioaccumulation. The fundamental difference is that the former requires transfer of contaminants between trophic levels via ingestion. In contrast bioaccumulation requires only uptake from the water independently by each trophic level and species.
Comprehensive reviews of metal contamination and accumulation in aquatic environments [Phillips, 1980; Grande and Andersen, 1983] have identified very few studies which demonstrate biomagnification of any of the heavy metals. Arsenic has been shown to increase through a marine food chain in a contaminated area near West Cyreenland, Table 2.7 [Bohn, 1975].

In contrast, other studies showed that no consistent increase along food chains whatever the metal studied. In Southampton Water and the Solent in the south of England there was no evidence of any increase of either arsenic or mercury at higher trophic levels [Leatherland et al, 1974].

Clearly biomagnification is very much the exception for metals but even without the amplification of contamination along the food chain, all trophic levels are contaminated. This is primarily due to direct uptake from the surrounding water [Brown, 1976; Hart, 1974].
<table>
<thead>
<tr>
<th></th>
<th>ppm dry weight as arsenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown seaweed</td>
<td>35.5</td>
</tr>
<tr>
<td>Zooplankton</td>
<td>6</td>
</tr>
<tr>
<td>Mussels</td>
<td>14-1-16.7</td>
</tr>
<tr>
<td>Prawns</td>
<td>62.9--80.2</td>
</tr>
<tr>
<td>Fish species</td>
<td>88.4 (43-4-188)</td>
</tr>
</tbody>
</table>
2.2.4 Toxicity of Heavy Metal to Activated Sludge in Sewage Treatment Plant

Besides the impact of heavy metal pollutants on human health and environmental ecosystems, the toxic effects of heavy metal on activated sludge processes in municipal sewage treatment plants have also received significant attention. Lester's work (1983) has already provided an extensive review on biological treatments. Toxic effects of heavy metals in wastewater treatment processes have been subjects of study for a long time. Previous studies showed that the presence of heavy metals even at sub-lethal level also affected the performance of COD removal efficiency in activated sludge process [Chua, et al, 1999]. The impacts of toxicity of heavy metals in activated sludge depends mainly on two factors, namely, metal species and concentration. A summary of effect of metal ions on activated sludge is summarised in Table 2.8.

The toxic effect of different concentrations of Ni²⁺ (5.0, 10.0 and 25.0 mg L⁻¹) on activated sludge was studied by Yetis and Gokeay (1989). For the concentration of 5.0 mg L⁻¹ of Ni²⁺, the removal efficiency was not adversely affected and the critical dilution rate was observed at 0.58 h⁻¹. For the concentration of 10.0 mg L⁻¹, the critical dilution rate was about 0.30 h⁻¹. For the concentration of 25.0 mg L⁻¹, the MLSS concentration was found to be a completely random manner. They showed that the removal efficiency of the activated sludge was not adversely affected by the presence of Ni²⁺ up to a concentration of 1.0 mg L⁻¹ while the feed solution contained 1300mg-COD L⁻¹. However, a concentration of 25.0 mg L⁻¹ Ni²⁺ reached toxic and lethal level and caused serious upsets in the system.

Dilek et al (1991) conducted a study on the effects of different concentrations of Cu²⁺ on organic removal efficiencies. They reported that removal efficiencies were not adversely affected when concentrations of copper ranged between 0.5 and 5 mg L⁻¹. Dilek and Yetis (1992) extended the study by Dilek et al (1991) on the removal efficiencies of various
heavy metals with different concentrations, i.e., Cu²⁺ (1.0, 5.0, 10.0, 25.0 and 50.0 mg L⁻¹), Ni²⁺ (5.0, 10.0 and 25.0 mg L⁻¹) and Cr⁶⁺ (1.0, 5.0, 10.0, 25.0 and 50.0 mg L⁻¹), using a laboratory-scale completely mixed activated sludge unit. Their results showed that removal efficiencies were not adversely affected by the concentration of Cu²⁺, Ni²⁺ and Cr⁶⁺ up to 10.0 mg L⁻¹, 10.0 mg L⁻¹ and 50.0 mg L⁻¹, respectively. For the Ni²⁺, a concentration of above 10 mg L⁻¹ led to unpredictable toxicity that caused oscillations in the rate of growth of microorganisms. According with the removal efficiencies, Lester et al. (1979) indicated that the metal toxicity in activated sludge processes can be expressed as follows: Pb>Cu>Zn>Cd, and Cr>Ni.

Apart from reduction of biomass, inhibition of BOD and COD removal efficiency, experimental data showed that the toxicity of heavy metal also result in deflocculation of sludge, decreased species diversity, changes in communities of microorganisms and inhibition of nitrification [Lester, 1983; Sujarittanonta and Sherurd, 1981]. Deflocculation can make the activated sludge unsettleable and therefore effecting treatment efficiency of sewage [Neufeld, 1976]. Cases in which severe shock-loading of heavy metals possessed ruinous effects in activated sludge systems have been reported [Lester, 1983]. Jackson and Brown (1970) indicated that the general toxicity ranking of heavy metals to activated sludge process is Ni²⁺>Zn²⁺>Cr⁶⁺>Cu²⁺>Cr³⁺, this result seemed to be different with Lester et al (1979). 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].
<table>
<thead>
<tr>
<th>Metal Ion</th>
<th>Observed Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag⁺</td>
<td>1 mg/L causes 2% inhibition, 10 mg/L cause 57% inhibition after two days (Ag as AgNO₃, in Sapromat); Limiting concentration for continuous input 1.2 mg/L.</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>Limiting concentration for continuous input 1.0 mg/L; Elevation of turbidity of treated effluent from 0.8 mg/L; Limiting concentration for shock loads (4 hrs with tₘₙ = 6 hrs) 50 mg/L for CuSO₄, 10 mg/L for copper cyanide complex; 10 mg/L causes 38% inhibition, 50 mg/L causes 85% inhibition of substrate respiration after five days (Cu²⁺ as CuSO₄, Sapromat).</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>Above 40 mg/L deflocculation occurs; Under continuous input, 5-10 mg/L no adverse effect; 20 mg/L causes inhibition; Limiting concentration for continuous input 5-10 mg/L 91.9% inhibition of substrate respiration for 20 mg/L after 24-48 hrs, 81.6% inhibition after 48-72 hrs, failing to 66.8% after 72-96 hrs and 45.7% after 96-120 hrs. Corresponding values for acclimatized sludge 46.6%, 2.8%, 16.0%, 17.6%.</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>Limiting concentration for continuous input 1-2.5 mg/L with continuous input of 2.5-10 mg/L, BOD removal efficiency is 5% lower; For continuous input at 10 mg/L, BOD₅ removal 90%, at 40 mg/L, BOD₅ removal = 83%, at 60 mg/L, BOD₅ removal very low.</td>
</tr>
<tr>
<td>Cr³⁺</td>
<td>Limiting concentration for continuous input: 10 mg/L BOD₅ removal drops to about 3%; For shock loading 10 mg/L no effect: at 100 mg/L deflocculation Deflocculation occurs from 20 mg/L.</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>Limiting concentration for continuous input: 10 mg/L Total inhibition of respiratory activity at 2000 mg/L.</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>At 1 mg/L substrate respiration after 5 days reduced by 5%, at 10 mg/L by 26%, at 20 mg/L by 43%, at 50 mg/L by 59% (Co³⁺ as CoCl₂ in Sapromat).</td>
</tr>
</tbody>
</table>
2.3: Conventional Methods for Heavy Metal Removal from Wastewater

Due to the toxicity of heavy metal to human, aquatic biota and activated sludge, pretreatment of metal-laden wastewaters before being discharged into water body is desirable. The most common methods for removing heavy metals from wastewater include chemical precipitation, ion exchange, carbon adsorption, membrane separation, electrodialysis treatment and leaching recovery. However, many of these methods may be ineffective or extremely expensive, especially when the metal concentration are relatively low (around 1-100mg L\(^{-1}\)) [Sag et al., 1995b]. The process descriptions and environmental and cost evaluation of these conventional treatment methods are summarised as follows:

2.3.1 Chemical Precipitation

Precipitation of metal-laden wastewaters involves the addition of chemicals to alter the physical state of the dissolved or suspended metals and to facilitate their removal through sedimentation. These precipitates may then be processed further for metals recovery. Typical chemicals often used to precipitate metals for aqueous streams include caustic soda, lime, sodium sulfide, ferrous sulfide, soda ash, phosphoric acid/sodium phosphate, and sodium borohydride. Because wastewaters containing complexed metals may not be effectively treated by such chemical additions, the use of reducing agents such as sulfur dioxide, ferrous sulfate, sodium borohydride, and sodium bisulfite may be required for their precipitation from the water matrix. Callodial suspensions require the use of coagulants/flocculants such as lime, alum, and synthetic polyelectrolytes for adequate precipitation. Precipitation, reduction, and coagulation/flocculation are followed by sedimentation (settling).
In well-run operations, chemical precipitation followed by sand filtration (for polishing) has been shown to meet current metal discharge guidelines for wastewaters. However, chemical precipitation of wastewater generates large volumes of sludges, which is harmful to the environment and more difficult to be treated than wastewater. It needs high disposal costs and land use. The limitation of chemical precipitation for treating heavy metal is about residual concentration of 1 mg L\(^{-1}\).

Approximate costs of capital investment for a 40 m\(^3\) h\(^{-1}\) treatment system is equivalent to about 15000 thousand HK dollars, and the cost for treating one ton metal-landen wastewater by chemical precipitation is equivalent to about 2.0 HK dollars, [Versar, Inc, 1984].

### 2.3.2 Electrodialysis

Electrodialysis, one of the more recent technologies applied to the recovery of plating chemicals from wastewater, uses an electric field as the driving force to remove charged ionic species from a feed stream. The exchange membranes allow appropriate ions to pass from the feed stream to a concentrated ionic solution (see Figure 2.1). Packaging several cell pairs of membranes between electrodes and manifolding the streams generates a concentrated stream and a depleted stream. Further ion reduction of the depleted stream can be accomplished in additional stages.

The environmental impacts are limited to those resulting from pretreatment and posttreatment. Pretreatment operations generate wastes such as spent filter elements, oil and grease, and sludge from scaling components. Post-treatment requirements are minimal, but they could involve treatment of process streams that accumulate contaminants in a near zero discharge system.
Typical capital costs of an electrodialysis systems for treatment of plating rinsewaters range from HK$ 400,000 to HK$ 800,000 based on the size and treatment capacity, [Higgins, 1985].

Operating costs depend on such factors as energy costs (e.g., electricity), maintenance requirements, replacement rates for parts (e.g., electrodes), and operating labor. The operating cost estimated by Higgins (1985) for a plant treating 20,000 tons yr\(^{-1}\) of metal-bearing waste is equivalent to about 3- 4.0 HK dollars per ton, which is more expensive than chemical precipitation system. Operating costs will vary depending on the quantity of the waste to be treated and the type and amount of metals contained in the waste.
Figure 2.1 Schematic diagram for electrodialysis

A --- Cation Permeable Membrane  C --- Anion Permeable Membrane

+ --- Cation  -- --- Anion
2.3.3 Membrane Separation

Microfiltration (MF) and ultrafiltration (UF) membranes cannot be applied directly to recover metals present as dissolved solids in wastewaters; therefore, MF and UF systems were used to separate precipitated metals from waste streams. Ultrafiltration can be used as a pretreatment method for RO units to avoid fouling of the RO membranes.

A typical MF or UF treatment system involves chemical precipitation followed by pressurized flow of the underflow through the filter membrane to remove the precipitate (see Figure 2.2). The pressure exerted on the solution forces clean solution through the membrane.

The principal environmental impact of MF/UF systems will result from the hazardous sludge generated by the process. Dewatering, followed by solidification or encapsulation, will be required. The cost of treatment and disposal, which will depend on the contaminant, could be significant.

The capital cost for the various sizes of standard advanced membrane filtration units range from HK$700000~2400000. Operation and maintenance costs, which depend greatly on waste composition, vary from HK $3 to HK $10 per tone of wastewater treated. These costs do not include costs for pretreatment chemicals, labor, and posttreatment [Selldorff, 1989].
Figure 2.2 Schematic diagram of precipitation/UF system for heavy metal removal
2.3.4 Adsorption

Activated carbon has been widely used for the removal of organics present in low levels (usually less than 1,000 mg L\(^{-1}\)) in contaminated liquids; however, also has been given to the removal of inorganics (e.g., metals). The process of metals removal by activated carbon often involves the use of multiple columns or tanks filled with carbon and operated in series or parallel configurations. Figure 2.3 is a schematic of column arrangements commonly applied in treating contaminated solutions. The carbon bed depth should be high enough to remove all the metals from the solution to the required concentration.

Environmental impacts of activated carbon adsorption are primarily associated with regeneration or disposal of the activated carbon. The carbon materials are regenerated using a strong acid such as sulfuric acid or base to remove adsorbed metals from the pores of the activated carbon.

Direct capital costs for carbon adsorption systems applicable to metals treatment include the purchase of equipment such as storage tanks, prefilters, carbon columns, waste feed pumps, piping materials, and automatic controls. Operation and maintenance costs include operating labor, electricity, and activated carbon consumption. The activated carbon is relatively expensive of 25-30 HK dollars/kg, and the operating costs for treating 10m\(^3\)/h metal containing wastewater is about 2.5~3.0 HK dollars [ICF, Inc, 1984].
Figure 2.3 Schematic diagram of adsorption process for heavy metal removal
2.3.5 Ion Exchange

Ion exchange is a technology applied in treating (1) metals in wastewaters where the metals are present as soluble ionic species (e.g., \( \text{Cr}^{3+} \) and \( \text{CrO}_4^{2-} \)); (2) nonmetallic anions such as halides, sulfates, nitrates, and cyanides; and (3) water-soluble, ionic organic compounds including (a) acids such as carboxyls, sulfonics, and some phenols, at a pH sufficiently alkaline to yield ionic species, (b) amines, when the solution acidity is sufficiently acid to form the corresponding acid salt, and (c) quaternary amines and alkyl-sulfates.

Ion exchange is a reversible chemical reaction in which an ion from solution is substituted for a similarly charged ion attached to an immobile solid particle. The use of this process is practical only on wastewaters and sludge leachates. In conventional ion exchange, metal ions from dilute wastewater solutions are exchanged for ions electrostatically held on the surface of the exchange medium. Ion exchange material can either be naturally occurring inorganic zeolites or synthetically produced organic resins. Synthetic resins are the predominant ion exchange medium used today because their characteristics can be tailored to specific applications [Versar, Inc. 1984].

Ion exchange can provide above 90 percent removal of metal ions from wastewaters and, as a final polishing system, yields satisfactory effluent discharges under current regulations [Cushnie, 1985]. Brine solutions created by regeneration are highly concentrated with metals; therefore, they could be recovered or returned to the process bath for reuse. Spent resins may need to be further treated prior to land disposal in order to comply with land disposal requirements.

The costs of ion exchange treatment per unit volume of wastewater decreases with a decrease in the concentration of the metal ion; therefore, ion exchange provides an
economically attractive means for treating wastewaters with metal concentrations of less than 200 mg L\(^{-1}\). (EPA-600/2-8—074, 1980). When a reduction in dissolved solids is of primary interest, ion exchange is considered practical economical if the solids concentration is less than 1000 mg L\(^{-1}\).

The capital cost of a copper recovery system using chelating ion exchange/electrowinning (50 gpm) is estimated to be approximately HK$10 million with operating costs of approximately HK$120,0000/yr. [Higgins and Gemmell, 1989]. The cost treating one tonne heavy metal containing wastewater is equivalent to about 2-3.0 HK dollars [Patterson, 1985].

According to above descriptions, in view of environmental, cost and efficient points, these conventional procedures have some significant disadvantages, namely, removing metal incompletely, high reagent requirement, generation of toxic sludge or very expensive when the contaminant concentrations are relatively low (around 1-100 mg L\(^{-1}\)) [Sag et al., 1995b]. Moreover, the use of ion exchange resins solely for purifying wastewaters is limited by the high price of materials though the cost may be partially offset by the value of the metal recovered. Otherwise, additional chemical dosage may impact the microorganism activity of subsequent activated sludge process for removal of COD.

Therefore, their application is still limited by economic or land technological reasons, including high operation cost, fouling of components, impractical at low metal concentration and uneconomical or non-selective metal recovery, etc. [Netzer and Reszedit, 1979; Cushnie, 1985; Patterson, 1987].
2.4 Biosorption for Heavy Metal Removal and Recovery

In the past decade, heavy metal removal by living/dead organisms or biological materials derived from them (which are thus commonly named as "biosorbents") has recently interested many researchers. It is because some biosorbents (biological materials used to adsorb metals) show high heavy metal binding capacity and the biological material is abundance and obtaining inexpensively from natural environment. Consequently, biosorbents are now being considered as an alternative to ion exchangers or other metal extraction and concentration operations in metal recovery [Volesky, 1987].

2.4.1 What is Biosorption

Volesky and Holan (1995) defined biosorption as the sorption and/or complexation of dissolved metals based on the chemical activity of microbial biomass. Muraleedharan et al. (1991) described biosorption as a process in which solids of natural origin, for example, microorganisms, alive or dead, or their derivatives, are employed for sequestration of heavy metals from an aqueous environment. In a narrow sense, the term "biosorption" is used to describe the passive (i.e., not metabolically mediated) accumulation of metals elements by biological materials, it should be distinguished from “bioaccumulation”, which is a just active process relying on metabolic activity and therefore occurs only in living organisms. Usually, for biosorption, both living and dead biomass can be served as a basis for biosorbents.

However, in broad sense, the term “biosorption” often indicate including both passive accumulation (biosorption) and metabolized-dependent accumulation (bioaccumulation). In natural environment, biosorption process for sequestration of heavy metal by microorganism cell often includes both passive accumulating and metabolized-dependent
accumulation.

The metabolized-dependent bioaccumulation of metals by living microorganism 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 metabolism-dependent living microbial cells could be termed "bioaccumulation" [Volesky 1987].

In addition, it was well known that all cell surface, such as algae, fungi, bacteria as well as their derivatives like extracellular polymers are anionic, therefore they could interact with cationic metallic species, [James, 1982]. Many studies showed that soluble metal ion in the environment could be captured by the cell wall because of reactive chemical site binding within its fabric, [Doyle et al., 1980; Beveridge et. al., 1982]. The uptake of metals on cell surface by metabolism-independent living/dead microbial cells could be termed "biosorption".

During the period of last two decades, bacteria, algae, fungi and yeast or their derivatives have been used successfully as biosorbent for heavy metal removal (including biosorption and bioaccumulation), bacteria are of particular interest because of their high cell surface area per volume, [Bakkaloglu, 1998; Gutnick et al., 2000; Kratochvil,1998; Mameri, 1999; Volesk, 1994; Wong et al.,1993a,b; Yetis,1998].

2.4.2 Mechanism of Biosorption

Biosorption for sequestration of heavy metal include metabolism-dependent accumulating process just by living cell biomass and passive accumulating process both by living/dead cell biomass. Both the uptake processes play roles in sequestration of
heavy metal in natural environment. A clear understanding of the biosorption mechanisms is very crucial for the development of microbial processes in order to concentrate, remove and recover rare, precious and strategic metals from aqueous wastes. For instance, if the chemical or physiological reactions of metal removal were known, the specification and process parameters could be controlled in order to change the rate, quantity and specificity of metal uptake [Muraleedharan et al. 1991]. Thus understanding the mechanism of biosorption is not merely a question of academic interest; practical benefits are also gained.

There are many ways for metal to be captured by the cell because of the complexity of the structure of microorganisms. Therefore, mechanisms of biosorption may vary in some cases and still not be very well understood. They may be classified by different criteria [Veglio and Beolchini, 1997].

Biosorption mechanisms can be classified into (1) metabolism dependent (bioaccumulation) and (2) metabolism independent (biosorption), according to the dependence on the cell's metabolism. Also, they can be classified into three categories: (1) extracellular accumulation/precipitation; (2) cell surface sorption/precipitation; and (3) intracellular accumulation, depending on the metal location after removal from the solution. Table 2.9 shows the various biosorption mechanisms classified according to both classifications [Veglio' and Beolchini, 1997].

Microbial cells accumulate heavy metals in a metabolism-dependent way by mainly three mechanisms: metabolism-mediated precipitation, redox reaction and transportation through ion transport systems.

Being metabolism independent, physical adsorption, precipitation, ion exchange and complexation can occur in either viable or non-viable microorganisms. These types of
physiochemical interaction between metal and cell rely mainly on the type of functional
groups present on the cell surface. Cell walls of microbial biomass mostly composed of
polysaccharides, proteins and phospholipids, which provide abundant binding sites such
as carboxylate, hydroxyl, sulphate, phosphate and amino groups for cationic metallic
species. This physiochemical process of metal biosorption is relatively rapid and can be
reversible [Kuyucak and Volesky, 1988].

The metal uptake mechanisms by metabolism-dependent accumulating process and
passive accumulating process are discussed below.
Table 2.9 Biosorption mechanisms. (a) Classified according to the dependence on the cell's metabolism. (b) Classified according to the location where the metal removed is found.

(a)

<table>
<thead>
<tr>
<th></th>
<th>Transport across cell membrane</th>
<th>Precipitation</th>
<th>Physical adsorption</th>
<th>Ion exchange</th>
<th>Complexation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolism dependent</td>
<td>✗</td>
<td>✗</td>
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<tr>
<td>Metabolism independent</td>
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(b)

<table>
<thead>
<tr>
<th></th>
<th>Transport across cell membrane</th>
<th>Precipitation</th>
<th>Physical adsorption</th>
<th>Ion exchange</th>
<th>Complexation</th>
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<tr>
<td>Extracellular accumulation/precipitation</td>
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<tr>
<td>Cell surface adsorption/precipitation</td>
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<tr>
<td>Intracellular Accumulation</td>
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</table>
Metabolism-Dependent Metal Uptake by Microbial Cells - Bioaccumulation

1: Metabolism-Mediated Precipitation of Heavy Metals

Many microorganisms are able to produce hydrogen sulfide which reacts 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 react with phosphate produced by bacterial cell to form precipitant. 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; Macasldhe 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]

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 Fe$^{2+}$ to obtain energy and deposit oxidized iron compounds on their long twisted stalks. Lactobacillus plantarum, Pseudomonas sp., 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].

A laboratory-scale reactor using growing cells of Gallionella ferruginea and a S. phaerotilus sp. for iron removal has been described [Viswanathan and Boettcher, 1991]. The iron-oxidizing bacteria grew and attached to a sand-gravel column where Fe$^{2+}$-containing groundwater was passed through. The Fe$^{2+}$ resulted from microbial oxidation was allowed to be further hydrolysed to insoluble Fe(OH)$_2$ in a detention tank and the effluent was filtered before discharge. The iron removal efficiency could reach 90% (effluent Fe$^{2+}$ concentration = c.a. 0.3 mg L$^{-1}$) after 14 days of operation. Chromate is reduced to insoluble Cr$^{3+}$ 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]. Cr$^{3+}$ compounds formed would be suspended in solution but whether they are also accumulated by the bacterial cells has not been 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].
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, when entered into 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 been reported.

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 and Strandberg (1985), biosorption of heavy metals is now generally referred to the non-directed physico-chemical interactions that occur between metal species dissolved in aqueous phase and cellular components of living or dead organism 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; Volesky and Holan, 1995].

The following review will focus on the passive biosorption mechanisms of microorganisms.

1. Ion exchange

While some authors consider only an exchange of electrostatically bound ions to be ion exchange, in this chapter we adopt a broader definition of this term. The term "ion exchange" is used when the charge of ions taken up equals the charge of ions released (so that the charge neutrality of the particle is maintained), regardless of whether these ions are bound electrostatically or by complexation. The driving force of ion exchange is mostly the attraction of the biosorbent for the solute (metal). Metals can be bound electrostatically or by complexation. Interactions between the solute (metal) and the
solvent (usually water) play a role in so far as less hydrophilic (and consequently less hydrated) molecules have a lower affinity for the liquid phase and are therefore sorbed more easily [Stumm and Morgan, 1970]. The importance of ion exchange in biosorption has frequently been reported. The amounts of ions from the natural environment (Na⁺, K⁺, Ca²⁺, Mg²⁺ and H⁺) and from biosorbent pretreatment (such as protonation) which are released during biosorption balance the heavy metal uptake by algae [Crist et al, 1990; Kuyucak and Volesky, 1989a; Schiewer and Volesky 1995], bacteria [Plette et al, 1993], fungi [Fourest and Roux, 1994; Treen et al, 1984], and peat [Chen et al, 1990; Crist et al, 1992].

Desorption can, in many cases, also be interpreted in terms of ion exchange. Such "competitive" desorption can be achieved by acids (e.g., HCl and H₂SO₄) and/or salt solutions (e.g., CaCl₂) [Aidor et al, 1995; Kuyucak and Volesky, 1989b]. In each case, the cation (H⁺, Ca²⁺) competes with the bound metal ion for the binding sites and replaces it if the concentration of the desorption agent is high enough.

2. Physical Adsorption and Precipitation

We use the terms of "adsorption" and "microprecipitation" to describe the accumulation of electrically neutral material which does not involve the release of a stoichiometric amount of previously bound ions. The difference between adsorption and microprecipitation is that in the former case, affinity between sorbent and sorbate (metal complex) and in the latter case limited solubility (i.e., an interaction between the solute and solvent) represents the main driving force. In microprecipitation, the metal cation and an anion (e.g., SO₄²⁻, S²⁻, oxalate, or HPO₄²⁻), itself often a metabolic product of certain biomass types, form insoluble aggregates (salts or complexes) such as sulfides,
carbonates, oxides, oxalates, and phosphonates [Beveridge, 1990; Rencle, 1990]. Changed local pH or redox potential can also influence the occurrence of precipitation. Microprecipitation does not necessarily involve a bond between biomass and metal. The process may, however, be nucleated by metal initially bound to active sites in the biomass [Mayers and Beveridge, 1989]. This means that a two-stage process takes place where binding to specific sites is followed by microprecipitation. The latter process is not limited by the number of binding sites but can occur in multiple layers [Macaskie et al, 1987b; Macaskie, 1990]. Sorption of neutral complexes is thought to be responsible for Cu binding to peat at high concentration [Chen et al, 1990].

3. Electrostatic Attraction and Complexation

Electro-negative charged ligands in the biomass (such as carboxyl groups) can react with metal ions to form complexes (or coordination compounds). Chelation, the binding of one metal ion to two coordinating atoms in the same biomolecule, may also occur in the above process. Complex formation involves both covalent and electrostatic components, whose relative contribution can be estimated by investigating how specific the binding is. When purely electrostatic attraction occurs, the binding strength should correlate with the charge density \((Z^2/\eta_{hyd})\). Ions of same charge \((z)\) and hydrated radius \((\eta_{hyd})\) should therefore be bound with equal strength. Major deviations of the binding strength from the \((Z^2/\eta_{hyd})\) correlation indicate a tendency toward a covalent bond character.

The nature of the ions released provides information about the bond type. Electrostatically bound ions cannot displace covalently bound ions. It was observed that proton release occurred only during heavy metal uptake, not during light metal uptake [Crist et al, 1981; Haug and Smidsrod, 1970]. Since protons are mainly bound covalently,
the binding of heavy metals must have been more covalent than that of light metal ions. Similarly, the more Na\(^+\) (which binds only electrostatically) reduces the uptake of other ions, the higher is the contribution of electrostatic attraction in the binding of those ions [Schiewer and Volesky, 1997].

Different correlations have been proposed to describe the increase of ionic-bond character with an increasing difference in the electronegativity between the two bonding atoms [Buffic, 1988; Dean, 1985]. For typical elements in biological ligands (O, N, and S), the ionic-bond character therefore increases with the electronegativity of the metal. It follows from the concept of hard and soft acids and bases that Pb\(^{2+}\) and Cu\(^{2+}\) are expected to display more covalent bond character and consequently stronger binding than are the hard ions Na\(^+\) or Ca\(^{2+}\).

**Binding Sites of Metal Ions in Cell Surface**

Numerous chemical groups have been proposed to contribute to biosorptive metal binding by, e.g., algae [Crist et al, 1981; Greene et al, 1987], bacteria [Brierley 1990], or biopolymers [Hunt, 1986], including hydroxyl, carbonyl, carboxyl, sulfhydryl, thioether, sulfonate, amine, imine, amide, imidazole, phosphonate, and phosphodiester groups. Whether any given group is important for biosorption of a certain metal by a certain biomass depends on factors such as the quantity of sites in the biosorbent material, the accessibility of the sites, the chemical state of the site (i.e., its availability), and the affinity between the site and the metal (i.e., the binding strength). For covalent metal binding, even an already occupied site is theoretically available. To what extent the site can be used by the metal in question depends on its binding strength and concentration compared to the metal already occupying the site. For electrostatic metal binding, a site is
available only if it is ionized.

The major binding sites in biosorption are acidic. Many groups (hydroxyl, carboxyl, sulphydryl, sulfonate, and phosphonate) are neutral when protonated and negatively charged when deprotonated. When the pH of the solution exceeds its pK\(_a\), these groups become mostly available for the attraction of cations. Amine, imine, amide, and imidazole groups, on the other hand, are neutral when deprotonated and positively charged when protonated. Therefore, they attract anions if the pH is lowered such that the groups are protonated. The structural formulae and pK\(_a\) values of binding groups are summarized in Table 2.10.

For a freshwater alga, *Chlorella*, the charge was positive (probably due to amine groups), favouring anion binding, at pH < 3 and negative (mostly carboxyl groups probably) at higher pHs, so that electrostatic attraction of cations occurred [Greene, 1987]. The charge of the biosorbent does not depend exclusively on the pH value. Covalent binding of metals can "consume" negatively charged groups. Groups become charge neutral that would otherwise have been negatively charged in metal-free solution of the same pH [Schiewer and Volesky, 1997].
Table 2.10 Structural formulae and pK values of binding groups in biomass

<table>
<thead>
<tr>
<th>Group</th>
<th>Structural formulae</th>
<th>pK&lt;sub&gt;a&lt;/sub&gt;</th>
<th>HSAB classifications</th>
<th>Ligated atom</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyl</td>
<td>-OH</td>
<td>9.5-13</td>
<td>Hard</td>
<td>O</td>
</tr>
<tr>
<td>Carboxyl (ester)</td>
<td>&gt;C=O</td>
<td></td>
<td>Hard</td>
<td>O</td>
</tr>
<tr>
<td>Carboxyl</td>
<td>-C=O</td>
<td>1.7-4.7</td>
<td>Hard</td>
<td>O</td>
</tr>
<tr>
<td>Sulfhydryl (thiol)</td>
<td>-SH</td>
<td>8.3-10.8</td>
<td>Soft</td>
<td>S</td>
</tr>
<tr>
<td>Thioether</td>
<td>&gt;S</td>
<td></td>
<td>Soft</td>
<td>S</td>
</tr>
<tr>
<td>Sulfonate</td>
<td>S=O</td>
<td>1.3</td>
<td>Hard</td>
<td>O</td>
</tr>
<tr>
<td>Amine</td>
<td>-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>9-11</td>
<td>Int.</td>
<td>N</td>
</tr>
<tr>
<td>Secondary amine</td>
<td>&gt;NH</td>
<td>13</td>
<td>Int.</td>
<td>N</td>
</tr>
<tr>
<td>Imine</td>
<td>=NH</td>
<td>11.6-12.6</td>
<td>Int.</td>
<td>N</td>
</tr>
<tr>
<td>Amide</td>
<td>-C=O</td>
<td></td>
<td>Int.</td>
<td>N</td>
</tr>
<tr>
<td>Imidazole</td>
<td>-C – N – N</td>
<td>6.0</td>
<td>Soft</td>
<td>N</td>
</tr>
<tr>
<td>Phosphonate</td>
<td>-P=O</td>
<td>0.9-2.1</td>
<td>Hard</td>
<td>O</td>
</tr>
<tr>
<td>Phosphodiester</td>
<td>&gt;P=O</td>
<td>1.5</td>
<td>Hard</td>
<td>O</td>
</tr>
</tbody>
</table>
2.4.3 Biosorbent Materials

A multitude of different biomaterials have been examined for their biosorptive properties, and different types of biomass have shown levels of metal uptake high enough (the order of 50-100 mg g⁻¹ or 0.5-1 mmol g⁻¹) to warrant further research [Weppen and Hornburg, 1995]. The three classes of biosorbents most frequently used are based on algal, bacterial, and fungal biomass. Other types of biosorbents include peat, pectic substances, and cellulosic materials (such as wood chips).

2.4.3.1 Evaluation of Biosorbent

To evaluate the suitability of biosorbent for field application in the treatment of metal-laden wastewaters, it is necessary to study the maximum removal capacity ($q_{\text{max}}$), kinetics of biosorption, metal recovery and regeneration as well as physical state of the biosorbent. Therefore, it is preferred to select biosorbents with the following properties [Volesky, 1987]:

1. the active biosorbent should be easily propagated, cheap and reusable;

2. the particle size, shape and mechanical properties should be suitable for use in a continuous-flow system in completely mixed, packed or fluidized-bed reactor configurations;

3. the uptake and release of the metal should be rapid and efficient;

4. the separation of the biosorbent from solution should be cheap, efficient and rapid;

5. the biosorbent should be metal-selective so as to separate the desired metal from a mixture of various metals solution;

6. the regeneration of metals from the biosorbent should preferably be metal-selective
and economically feasible, and the biosorbent should be in a physical state that can be used.

The metal binding of three major classes of biosorbents, algae, bacteria and fungi was evaluated, and the cell structure and composition of algae, bacteria and fungi, and their roles on heavy metal binding were discussed as follows:

**Algae**

The abundance of algae should not be underestimated. A total of 90% of all photosynthesis occurs through algae, and the mass of algal cell wall polymers alone equals about half of the total nonaqueous biomass [Siegel and Siegel II, 1973]. The world harvest of seaweeds for food and algal products (e.g., agar, alginate, which can be employed as thickeners) already exceeds 3 million tons annually [Macaskie, 1991] and potential harvests are estimated as 2.6 million tons for red algae and 16 million tons for brown algae [Chapman 1980]. Apart from being readily available, the advantages of algal biosorbents are that the particles can be large enough to eliminate the need for immobilization.

Biosorption in algae has been attributed mainly to the cell wall, where both electrostatic attraction and complexation can play a role. The most common types of algae that possess complete cell walls are Rhodophyta (red algae), Chlorophyta (green algae), and Phaeophyta (brown algae) [Bold et al, 1987]. A typical algal cell wall consists of a fibrillar skeleton and an amorphous embedding matrix (Figure 2.4). The most common fibrillar material is cellulose, but it can be replaced by mannan or xylan in some green algae and in some genera of red algae. Brown and red algae contain the largest amounts of amorphous matrix polysaccharides. While alginic acid is the dominant constituent,
followed by the sulfated polysaccharides such as fucoidan, in the brown algal matrix, a number of sulfated galactans (agar, carrageenan, porphyran, etc.) plays a major role in red algae. The green algal matrix consists of complex heteropolysaccharides (involving galactose, arabinose, xylose, rhamnose, and glucuronic acid) that may be sulfated [Lee, 1989].

The main acidic groups responsible for metal uptake are the carboxyl groups of uronic acids (guluronic, mannuronic, and glucuronic) as well as sulfonate groups. Hydroxyl groups are present in all polysaccharides, but they become negatively charged only at pH > 10, at lower pHS, they play only a secondary role. Carboxyl groups can be the dominating binding groups, especially in brown algae. This is the case because alginic acid, which is composed of mannuronic and guluronic acids (Figure 2.5a), can constitute up to 40% of the dry weight of brown algae [Pereival and McDowell, 1967]. Carboxyl groups are found to a lesser extent in the glucuronic acid of green algal matrix polysaccharides and red algal agarpectin [Siegel and Siegel II, 1973]. Sulfonate groups are abundant in red algae due to their high content of carrageenan (Figure 2.5b), porphyran, and agar (up to 70% of the cell wall [Bold and Wynne, 1985]), but they also occur in fucoidan of brown algae and sulfated heteropolysaccharides of green algae. Particularly for green algae, the contribution of functional groups of amino acids (hydroxyl, carboxyl, sulfhydryl, amine, imine, amide, and imidazole) [Bailey and Oillis, 1986] should not be neglected: protein can constitute 10 to 70% of the (outer cuticular layer) [Bold and Wynne, 1985] of the cell wall [Siegel and Siegel II, 1973].
Figure 2.4 Cell wall structure in algae

ALGAE (example: brown algae)

outside

Mucllage
Cell Wall
Plasma Membrane

inside

Alginate & Fucoidan Matrix
Cellulose Fibres
Protein
Phospholipid
Figure 2.5 Structures of important biomolecules involved in metal binding in algae.
Bacteria

Bacterial biomass (e.g., *Bacillus, Streptomycyes, and Citrobacter*) can be economically obtained from fermentation industries, activated sludge process.

Microprecipitation is a common phenomenon in metal binding by bacteria [Mclean and Beveridge, 1990], but complexation by extracellular substances or by N and O ligands in the cell wall, as well as electrostatic attraction to charged groups in the cell wall, may also occur [Brierley, 1990]. Microprecipitation is often preceded by binding to specific sites, which provides nucleation points [Mclean and Beveridge, 1990].

The higher metal binding capacity of gram-positive than of gram-negative bacteria can be traced back to their cell wall makeup (Figure 2.6): gram-positive bacteria possess a thicker peptidoglycan (PG) layer [Brierley, 1990].

The gram-positive bacterial cell wall features a ca. 20 to 30 nm-thick layer (ca. 25 molecules) of PEG into which teichoic acids (TA) and teichuronic acids (TUA) are embedded [Beveridge, 1986; Mclean and Beveridge, 1990]. The total cell wall can be 50 to 150 nm thick [Remacle, 1990]. PEG is reported to represent 40 to 90% of the cell wall [Remacle, 1990]; other sources mention that TA and TUA can constitute up to 80% of the wall [Beveridge, 1986]. PEG is a linear polymer of alternating glucosamine and muramic acid with several peptide side chains (Figure 2.7a,b). These side chains bear one carboxyl group at the terminal amino acid and additional functional groups on certain intermediate amino acids like asparagine, lysine, cysteine, or aspartic acid [Mclean and Beveridge, 1990]. TA contains phosphodiesters (Figure 2.7c,d), and TUA feature carboxyl groups; both of these contribute to the negative charge of the biomass and enable ion exchange [Brierley, 1990]. For *Bacillus subtilis*, the major importance of the carboxyl groups of the PEG as well as the minor contribution of phosphate groups in metal uptake was
demonstrated by blocking experiments. Amine groups did not appear to be relevant [Mclean and Beveridge, 1990].

Gram-negative bacteria have a much thinner PEG layer are to three molecules thick [May, 1984] (seeing Figure 2.6), which makes up about 10% of the weight of the total cell wall, which can be 30 to 80 nm thick [Schiewer and Volesky, 1995]. The PEG layer of gram-negative bacteria does not contain TA or TUA. Therefore, they offer less negatively charged carboxyl groups which is a reason for their lower biosorptive capacity [Brierley, 1990; Remacle, 1990]. On the other hand, a characteristic of these bacteria is an outer membrane which contains lipdopolysaccharides and phospholipids. Their phosphonate groups, which create a negative surface charge conducive to cations binding, were confirmed to be the primary metal binding site in *Escherichia coli* (Beveridge, 1986; Mclean and Beveridge, 1990).

Proteinaceous surface arrays, or S-layers, are present in many bacteria [Beveridge, 1986; Mclean and Beveridge, 1990]. Extracellular polymers of the capsule or slime layer contain carboxyl and occasionally phosphonate or sulfonate groups [Mclean and Beveridge, 1990]. Certain bacteria produce $\text{SO}_4^{2-}$ or $\text{S}^{2-}$ or enzymes that liberate $\text{HPO}_4^{2-}$. These ligands may form microprecipitates with metal cations [Brierley, 1990; Mclean and Beveridge, 1990]. As in the case of bacteria, released metabolises may lead to microprecipitation (oxalates due to oxalic acid, sulfides due to $\text{H}_2\text{S}$) or chelation (citric acid, siderophores).
Figure 2.6 Cell wall structure in bacteria

GRAM-POSITIVE BACTERIUM

Surface Array
Cell Wall
Plasma Membrane

outside

Protein
Peptidoglycan Fibres
Teichuronic & Teichoic Acids
Phospholipid

inside

GRAM NEGATIVE BACTERIA

Surface Array
Outer Membrane
Cell Wall
Plasma Membrane

outside

Lipoplysaccharide
Lipoprotein
Peptidoglycan Fibres
Phospholipid
Protein

inside
Figure 2.7 Structures of important biomolecules involved in metal binding in Gram-positive bacteria.
Fungi

Fungi can be economically developed from industrial waste products. *Aspergillus niger* is used in the production of citric acid and of the enzyme glucamylase, *Saccharomyces cerevisiae* is used in the food and beverage industry, and *Rhizopus arrhizus* produces the enzyme lipase, just to name a few examples of fungi and yeasts that have been employed in biosorption studies [Deacon, 1984; Gadd, 1990; May, 1984]. Some filamentous fungi such as *A. niger* grow as pellets, which aids the recovery of the metal-laden biosorbent [Gadd, 1990]. Other types of fungi create problems of solid/liquid separation and are not easily filterable [Volesky, 1990a]. Another potential disadvantage of the use of fungi is that of impurities due to adherence of fermentation broth residue that may affect metal uptake.

Similarly to algae and bacteria, cell wall of fungi is the main location of metal deposition [Volesky, 1990a]. Polysaccharides constitute up to 90% of the cell wall of fungi [Remacle, 1990].

Figure 2.8 shows the fungal cell wall architecture. The inner microfibrillar layer of the wall usually consists of chitin (Figure 2.9), but cellulose or, in rarer cases, noncellulosic β-glucan (in Hemiascomyeeses, e.g., *Saccharomyces*) can take its place, depending on the taxonomic group [Mueller and Loeffler, 1976; Remacle, 1990]. The outer, more amorphous layer is made up of mostly α-glucans but can also contain mannan, galactans, chitosan (Figure 2.9) (Zygomycetes, e.g., *Mucor* and *Rhizopus*), or glycogen [Remacle, 1990]. As seen by a comparison of Figure 2.9, chitin is acetylated chitosan.

Phosphated polysaccharides may occur [Gadd, 1990]; the phosphate content in *Mucor* can exceed 20% of the cell wall dry weight [Volesky, 1990a]. The phosphate and carboxyl groups (of glueuronic acid) are thought to be responsible for the negative charge.
in the fungal wall, whereas the amine groups of the chitosan create a positive charge [Remacle, 1990; Volesky, 1990a]. Apart from electrostatic attraction to these charged groups, complexation with N or O donors (e.g., of chitin) may occur [May, 1984]. Since the fungal cell wall comprise of about is only about 10% proteins [Deacon, 1984; Volesky, 1990a], the importance of amino acid functional groups in metal uptake is relatively insignificant.
**Figure 2.8** Cell wall Structure of in fungi

**FUNGI** (example: type V, e.g. Euascomycetes)

- **outside**
  - Mucilage
  - Cell Wall
  - Plasma Membrane

- **inside**
  - Glucan Matrix
  - Protein
  - Chitin Fibres
  - Phospholipid
Figure 2.9 Structure of important biomolecules involved in metal binding in fungi cell wall
2.4.3.2 Screening Methods for Biosorbent with High Metal Binding Capacity

Biosorption for heavy metal and related topics often involves finding microorganisms with an extraordinary capability in accumulating heavy metals. Checking the efficiency of laboratory strains as well as the isolation of microorganisms from natural environment or habitats is often among the basic tasks to be performed. In order to facilitate and accelerate these tasks, some rapid screening methods for isolation of metal accumulating microorganism have been developed. Thomas et al (1995) developed an agar-plate screening method which enables the selection of high efficient silver, copper and nickle-accumulating microorganisms from several dozens of colonies. For selecting metal-accumulating microorganism, agar plates with well-developed colonies are overlayed with a further agar layer containing the metal salts of interest. After incubation the metal is visualized by precipitation with H2S solution and optical effects are interpreted. The main effects are the staining of colonies due to the accumulation and precipitation of metal and the formation of light haloes around the colony with the uniformly darkened agar as a result of the diffusion of dissolved metal towards the organisms.

Numerous literature has reported the positive and negative correlations between the heavy metal accumulation capacity of microorganisms and their respective resistance or tolerance to heavy metal [Boularbah et al, 1992; Cooksey and Azad, 1992; Han et al, 1992; Mergeay, 1991; Nies, 1992; Slawson et al, 1992; Wnorowski, 1991]. Selection of the microorganisms to be subjected to the test mainly depends on the purpose of the study to be performed. No general instructions can be supplied on where and how to find potent microorganisms, e.g. whether screening should start with a heavy metal contaminated site or with a common soil.

The choice of selecting medium depends on the target group of organisms and subsequent aims. For practical reasons the isolation of microorganisms needing vitamins or other
expensive compounds is to be avoided in many cases. For biotechnological applications it may be appropriate to take into consideration the availability of cheap waste products. Further adjustable parameters for pre-selection are medium pH, temperature, and the presence of oxygen or toxic substances such as antibiotics or heavy metals. Extra care has to be taken in the chemical composition of the medium due to complexation or precipitation of the metal of interest. In general the contents of phosphates, organic acids, complexing buffers, and complex nitrogen sources should be minimized for most heavy metals.

Besides the choice of the element, its speciation which is mainly determined by the pH, the ionic strength and the selected compound, are also of great importance [Mergeay, 1991; Morel, 1983; Sigg and Stumm, 1989]. The time of exposure is very important as it permits a distinction to be made between passive biosorption and additional active bioaccumulation. Biosorption usually proceeds within several minutes (about 15-20 min are necessary to gain a light halo due to diffusion), whereas bioaccumulation takes hours and may be seen after one or two days. Useful concentrations to give a uniformly coloured agar and sufficiently stained colonies are in the range of 1mM to 10 mM and mainly depend on the metal and on the recipe of the nutrient agar (Table 2.11).
<table>
<thead>
<tr>
<th>Metal</th>
<th>Compound</th>
<th>Concentration range [mM]</th>
<th>Precipitation with</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>AgNO$_3$</td>
<td>5-8</td>
<td>H$_2$S</td>
</tr>
<tr>
<td>Tl</td>
<td>TlNO$_3$</td>
<td>3-7</td>
<td>H$_2$S</td>
</tr>
<tr>
<td>Cu</td>
<td>Cu(NO$_3$)$_2$</td>
<td>6-10</td>
<td>H$_2$S</td>
</tr>
<tr>
<td>Ni</td>
<td>Ni(NO$_3$)$_2$</td>
<td>3-7</td>
<td>2% (NH$_4$)$_2$S</td>
</tr>
<tr>
<td>Pb</td>
<td>Pb(NO$_3$)$_2$</td>
<td>2-6</td>
<td>H$_2$S</td>
</tr>
<tr>
<td>Cd</td>
<td>Cd(NO$_3$)$_2$</td>
<td>1-4</td>
<td>H$_2$S</td>
</tr>
</tbody>
</table>
2.4.3.3 Living and Dead Cells for Biosorbent

Both living and dead cells can be used as biosorbent for heavy metal binding. There are distinct advantages in using either living or dead biomass [Brierley, 1990; Gadd, 1990; Macaskie, 1991]. The main advantages of using living biomass are that it is self-renewing, that active transport into the cell may lead to higher metal uptake levels, and that excreted metabolic products can contribute to metal removal. However, it was observed that in terms of the quantity of metal accumulated, the effects of dead and living biomass are comparable. The use of dead biomass avoids problems with toxicity (the toxicity of metals is often the very reason for using biosorption). Costs of cultivation of live biomass (e.g., nutrient supply) far exceed the costs of obtaining nonliving biomass. Waste products from fermentation processes or naturally abundant biomass can be used. Biosorption by dead biomass is often faster, since only the cell wall-based binding, not active transport into the cell, occurs. Another advantage of using dead biomass is the easier and nondestructive recovery of bound metals, which allows regeneration of the biosorbent material. Metals accumulated intracellularly by living biomass can often be recovered only when the cell is destroyed. In most cases, working with dead biomass offers more advantages and is therefore the object of the majority of more practically oriented biosorption studies. Wase and Forster, (1997) concluded that the use of dead cells offer the following advantages over living cells:

1. the metal removal system is not subject to toxicity limitations

2. no requirements for growth media and nutrients

3. biosorbed metal ions can be easily desorbed and biomass can be re-used

4. raw biomass can be obtained from the industrial fermentation industries

5. biomass can be pretreated to enhance the metal biosorptive capacity
6. much simpler process control

7. no concern over the disposal of metabolic products or surplus nutrients which would be required for a system using living cells

8. the biomass can be stored for a period of time
2.4.4 Applications of Biosorbnet for Heavy Metal Removal

Biosorption can concentrate metals for several thousandfolds: the factor by which the metal concentration in algae exceeds that in the surrounding seawater was as high as a few thousand [Black and Mitchell, 1952]. Therefore, biosorption could serve as a means of obtaining precious metals from the seawater or from processing solutions. Its main target is, however, to remove heavy metals which can be quite toxic even at low concentrations. Biosorption is particularly suited as a polishing step whereby wastewater with a low to medium initial metal concentration (from a few to about 100 ppm) is purified such that drinking-water quality is obtained. Biosorptive treatment of wastewaters with very high initial metal concentrations would lead to rapid exhaustion of the biosorbent material and thus require large amounts of biosorbent. Therefore, pretreatment of such effluents using other techniques, such as precipitation (which is currently used for 90% of heavy metal removal from industrial wastewater) or electrolytic recovery, may be more economical. On the other hand, advantages of biosorption (compared to precipitation) are that it offers high effluent quality and avoids the generation of toxic sludges [Brierley et al. 1986].

In general, biosorption is used to treat wastewater just containing relatively low concentration of heavy metal. Thus, as a polishing treatment technique, biosorption has to compete with other processes such as ion exchange, activated-carbon sorption, or membrane technologies (electrodialysis, reverse osmosis) that are currently used for this purpose. The advantages of biosorption are that it can be used under a broad range of operating conditions (pH, temperature, metal concentration, other ions in solution) and, especially, that it is cost-effective [Kuyucak, 1990]. Raw materials of a cheap cost can be used like suitable naturally abundant biomass types (e.g., certain seaweeds) or industrial waste by-product biomass (e.g., bacteria and fungi from the fermentation industry). In the
latter case, an additional benefit is that waste from one industry can be used to clean the waste from other processes. Moreover, very high cost-effectiveness can be achieved through the regeneration of biosorbents with the possibility of recovering the metal.

For example, a "Meander" system used at the Homestake lead mine (MO, USA) passes effluents containing Pb, Cu, Zn, Ni, Fe and Cd ions through various channels containing cyanobacteria and algae. Metals are removed from the water column with an efficiency greater than 99% [Jennett and Wixson, 1983; Erlich and Brierley, 1990]. Such complex systems likely utilize mechanisms, such as precipitation and entrapment of particulates, in addition to biosorption. The combined processes ultimately concentrate the metals in the sediment in forms which greatly reduce environmental mobility and biological availability [Brierley and Brierley, 1993].

Many microorganisms produce extracellular polymers or possess a capsule composed mostly of polysaccharide material or a slime layer. For example, Zooglea spp. produce copious amounts of a gelatinous polymeric matrix into which cells become embedded as they grow, and this layer also binds metal ions tightly. Zooglea forms a floc during conventional sewage treatment, and bound metals settle out along with the sludge [Hughes and Poole, 1989]. Metal ion binding correlates well with the anionic character of the biopolymers. Other bacterial surface polymers have been shown to serve as ligands for metal ions, including those of Klebsiella Enterobacter aerogenes [Rudd et al., 1984]. Arthrobacter viscosus [Scott and Pahner, 1986], and Pseudomonas sp. [Gadd and White, 1993].

During the two past decades, freely suspended fungi and yeast [Azab et al., 1990; Leuf et al., 1991; Volosky et al., 1993; Volosky and May-Phillips, 1995; Purnik et al., 1995; Dcy et al., 1995; Sag and Kutsal, 1996], have received more attention in connection with nonimmobilized metal biosorption systems, simply because plentiful amounts of waste
fungal biomass are generated as by-products of several types of industrial alcohol and antibiotic fermentations [Volesky, 1990b; Volesky and Holan, 1995; Omar and Merroun, 1996]. One such system obtained from *Aspergillus niger* waste from citric acid fermentations was shown to remove zinc, magnetite, and metal sulﬁdes from wastewater [Wainwright et al., 1990; Singleton et al., 1990]. This process was independent of metabolism but was favoured by growth, with the particles eventually becoming entrapped within the hyphal matrix. Al-Ashesh and Duvinjak (1995) demonstrated that living mycelia of *Aspergillus carbonarius* was able to adsorb copper and chromium and noted that an increase in uptake corresponded with an increase in pH. Treatment with alkali of *Bacillus sp.* wastes from industrial fermentations enhanced nonspeciﬁc metal uptake and drying, pelleting and chemically cross-linking of the biosorbent provided for indeﬁnite shelf life [Brierley, 1990]. Metal loads up to 10% of the dry weight of the bacterial wastes and over 99% removal of heavy metals, nonspeciﬁcally were documented. Effluents with 10-15 ppb metal were the norm. Metals were removed from the biosorbent using diluted sulfuric acid, NaOH, or complexing agents and recovered using standard chemical methods. Regeneration of the granules was by alkali treatment. Lead was found to adsorb at pH 5.5, to a nonliving biomass of *Saccharomyces uvarum* to 48.9 mg Pb g⁻¹ dry wt. biomass. Given that carboxyl and amine groups served as the ligand to which the metal bound, it was assumed that chitin was the most likely provider of these groups [Ashkenazy et al., 1997].

A proprietary method using processed granules of *Rhizopus arrhizus* was proved to be successful in immobilizing uranium ion [Tsezos and Deutschmann, 1990] at 50 mg U g⁻¹ biomass dry weight, in batch uptake studies. Complete uranium removal was demonstrated for dilute uranium ore bioleaching solutions (< 300 mg L⁻¹) with eluate concentrations after desorption approaching 5,000 mg U L⁻¹. A nonviable preparation of a
thermotolerant ethanol producing strain of *Kluyveromyces marxianus* was found to rapidly take up uranium (VI) with an efficiency of up to 150 mg U g\(^{-1}\) dry wt. biomass, but with lower binding at lower pH values [Bustard et al., 1997]. The bacterial exopolysaccharide of *Actinobacter RAG-1* has been shown to bind up to 240 mg U (as UO\(_2\)) g\(^{-1}\) biomass. Other exopolysaccharides of *Pseudomonas, Arthrobacter* and *Klebsiella* have been shown to be able to sorb up to 96 mg U g\(^{-1}\) [Veglio and Beolchini, 1997]. Bakkaloglu (1998), Mameri (1999), Wong et al. (1993a) demonstrated that Zn\(^{2+}\), Cu\(^{2+}\) and Ni\(^{2+}\) could be accumulated and removed efficiently from wastewater by living or dead bacterial cell biomass. Hu et al. (1996) demonstrated the utility of *Pseudomonas aeruginosa* immobilized in a novel polyurethane gel matrix, and developed a comprehensive mathematical model for the kinetics of uranium biosorption in a batch stirred tank reactor.

The abundant brewery and bakery yeast *Saccharomyces cerevisiae* has been the object of numerous biosorption studies. Yetis (1998), Wilhelmi and Duncan (1995, 1996) showed that yeast cells had the capability to adsorb Cu, Co, Cd, Ni, Zn and Cr with an uptake averaging around 40 µmol g\(^{-1}\) in continuous flow packed bed columns through eight repeated adsorption-desorption cycles.

It is evident that even with current technology, biosorption processes utilizing whole biomass is of high potential to replace existing metal-removal technologies, or it is an effective polishing unit for an existing treatment facility [Kapoor and Viraraghavan, 1995; Voleska and Holan, 1995].

### 2.4.5 Factors Affecting Biosorption of Heavy Metal

Numerous studies showed that effectiveness of biosorption could be affected by internal factors (cell structure and component) and external factors (environmental conditions).
Cell structure and component could be changed by, namely, cell growth age, cultural conditions of the microbial cells and pretreatment of the microbial cells before biosorption. External factors, or rather, environmental conditions of the biosorption process include pH, metal concentration, other cations, anions and temperature of wastewater solution.

2.4.5.1 Effect of Cell Internal Factors on Biosorption of Heavy Metal

1. Effect of Cell Cultural Condition on Adsorption Capacity of Biomass

Since most biosorption processes involve the microbial cell surfaces. When change the culture conditions of microbial cells like growth rate, medium composition, culture pH, oxygen and carbon dioxide concentrations, incubation temperature etc., the cell surface properties and components such as the content of protein, PEG, chitin, phospholipid and lipopolysaccharide would be influenced [Hancock and Poxton, 1988]. Thus it is reasonable to expect that some of these conditions can affect the biosorptive properties of microbial cells due to change cell surface structure and components.

For example, medium composition can influence the 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 was found to affect the cell surface composition of Aspergillus oryzae. C/N ratio of 10 -15 was 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 [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 an effective biosorbent for metals, a decreased pH might dissociate the trace elements from the biomass and increased their availability to the fungus.

2. Effect of Cell Growth Age on Adsorption Capacity of Biomass

The age of culture of microbial cells has been found to be one of the important factors affecting the biosorption properties of cell biomass. 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 increased after 20 hours of incubation [Treen-Sears et al., 1984b].

3. Effect of Pretreatment of Microbial Cells on Adsorption Capacity of Cell Biomass

One of the main aims of the pretreatment of biomass is to enhance the metal removal capacities of the biosorbents. Various physico-chemical pretreatments of microbial cells have been investigated for their effects on their subsequent biosorptive properties. Physical methods include vacuum and freeze-drying, boiling, autoclaving and mechanical disruption. Chemical methods include contacting the biomass with various organic and inorganic compounds [Siegel et al., 1990; Kapoor and Viraraghavan, 1995].

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 *R 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, namely, altering cell surface structure and properties of active groups, removing impurities on cell surface. 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.

2.4.5.2 Effect of Environmental Conditions on Biosorption of Heavy Metal

Although biosorption may be used under a wide range of operating conditions, biosorption involves certain physico-chemical interactions between the metals and the cellular components that is influenced by environmental conditions [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. Whereby the effect of environmental parameters on metal uptake were discussed below.
1. Initial Metal Concentration

One of the most important factors influencing metal uptake is, not surprisingly, the concentration of the metal itself. Biosorption can be applied in a broad range of metal concentrations. With increasing equilibrium metal concentration \([M]\), the metal uptake increases too. Whereas metal accumulation can occur in multiple layers in microprecipitation, leading to very high uptake values, a plateau value, \(M_{q_{\text{max}}}\) of the metal uptake is usually reached at high metal concentrations in sorption or ion exchange. The sorption capacity is limited by the number of binding sites in the biomass. At low metal concentrations, as occur in effluents from mining industry (which may contain less than 5 ppm of metal), the capacity of the biosorbent is not fully used.

2. Temperature

Sorption is often temperature dependent. Therefore, uptake values for different conditions are comparable only if they are obtained at similar temperatures (as implied in the term "sorption isotherm"). Simple physical sorption is generally exothermic, i.e., the equilibrium constants decrease with increasing temperature [Smith, 1981].

Since biosorption usually happens in a narrow temperature range (5 to 40°C), temperature effects are only of secondary importance. When the temperature was increased from about 5 to about 50°C, the equilibrium metal uptake increased only by a factor of about 2 or less [Kuyucak, 1990; Greene, 1988].

3. pH Value

Biosorption can be happened at a wide range of pH, from the low pH of acid mine
drainage to the high pH of wastewaters pretreated by precipitation. Since sorption can dramatically change with pH, this key parameter should always be reported. Data are comparable only if they are obtained at the same pH (e.g., all points on a sorption isotherm should be at the same pH).

There are several ways in which pH may influence sorption. First, the speciation of the metal in solution is pH dependent. While many metals occur as free hydrated species at lower pH, hydroxide form with increasing pH and eventually precipitation may occur. Sorption increases with decreasing solubility of the sorbate. Consequently, hydrolysed metal ions may sorb better than the free metal ion, because they are less hydrophilic, so that less energy is necessary for removal or reorientation of the hydrated water molecules of hydrolysed metals [Collins and Stotzky, 1992; Pagenkopf, 1978; Stumm and Morgan, 1970]. Second, extreme pH values can damage the structure of the sorbent, as could be seen in some electron micrographs of distorted cells reported in the literature [Kuyucak and Volesky, 1989b]. The third and often most important factor is pH can change the state of the active binding sites. Their protonation and consequently their availability can change dramatically if the pH is varied by 1 or 2 units. Cation binding usually increases with increasing pH. This occurs because negatively charged free sites allow electrostatic attraction of cations and, if the metal is covalently bound, high pH means less competition with protons for the binding sites (few sites are occupied by protons).

Nevertheless, it may not be appropriate to choose higher pH values for biosorption because the cell structure may be destroyed and the precipitation of the metal may occur. On the laboratory scale, this complicates the determination of the actual metal removal by binding to the biomass. On an industrial scale, precipitation may be desirable because it increases the overall removal of metal from the solution. However, it is possible that precipitates will clog the fixed-bed biosorption column.
4. Other Cations

Light metals (e.g., Na and Ca) frequently occur in wastewaters, especially after pretreatment by precipitation. Moreover, industrial wastewaters may contain more than one type of heavy metal. Similarly to protons, other cations can compete with the metal ion in question for binding. Therefore, the uptake of each metal cation is generally reduced, in the presence of others. This competition can occur at the level of both electrostatic and covalent binding.

Generally speaking, ions that are bound only weakly through mostly electrostatic attraction, like the alkaline (earth) metals, are effective in competing only with other weakly bound ions such as Zn$^{2+}$ [Ferguson, 1974]. To a lesser extent, they are able to reduce the uptake of ions like Cu$^{2+}$, Pb$^{2+}$ and Ag$^+$, where the bond character is more covalent [Ferguson, 1974; Tobin et al, 1988]. This is because light metal ions such as Na$^{2+}$ are able to compete only with the electrostatically bound fraction of a heavy metal ion such as Cd$^{2+}$. To the degree that Cd$^{2+}$ is covalently bound, it cannot be replaced by Na$^+$ [Schiewer and Volesky, 1997].

Among heavy metal ions, the effect of a more strongly binding ion such as Cu$^{2+}$ on the uptake of the weaker competitor such as Zn$^{2+}$ is obviously more pronounced than vice versa [Chong and Volesky, 1995]. Radioactive elements (such as uranium and radium) can be strongly binding and therefore radioactive, and heavy metal ions mutually influence each other's uptake [Tobin et al, 1988; Tsezos, 1986]. For competition in covalent binding, the charge of the metal is irrelevant. The level of competition depends mainly on whether two metals use the same binding sites and how strongly they are bound to any given site.
5. Anions

Anions of metal salts that balance the positive charge of metal ions are commonly occurred in metal-bearing solution. Additionally, chelating agents may be added in electroplating processes [Brierley, 1990]. These anions may affect the metal uptake. Anions could theoretically bind to the active sites of the biomass, thereby changing their state such that metal uptake is affected. However, there is little evidence of such phenomena. The more important effect of anions is that they may form complexes with the metals in solution. Whether this has a positive or negative effect on overall metal sorption depends on whether the metal-ligand complex has a higher or lower affinity for the biomass than the free cation does. In most cases of biosorption, metal uptake is reduced at increased ligand concentrations [Greene et al, 1986; Kuyucak and Volesky, 1989c; Tobin et al, 1987], which indicates that many complexes bind less strongly than the free metal ions. This effect is most pronounced for strong complexing agents such as EDTA [Ramelow et al, 1992; Tobin et al, 1987]. Most anions (e.g., sufonate, phosphonate, chloride, and carbonate) show only a weak influence to heavy metal binding by biosorbent [Tobin et al, 1987]. Consequently, the influence of anions is usually minor unless complex formation occurs.

2.4.6 Metal Desorption and Biosorbent Regeneration

In industrial applications of biosorption, the sequestered metal ions need to be recovered in a concentrated solution, simultaneously regenerating the biosorbent for additional use. This is the same operation as in the elution of conventional ion-exchange resins. The elution of the sequestered metal ions from the microbial biomass and the regeneration of biosorbent have not been studied as extensively as the respective uptake.
There are generally two avenues of dealing with the metal-laden biosorbent: either it must be disposed of (e.g., by incineration or deposition in landfills) or, preferably, it is regenerated. In the first case, biosorption serves to reduce the volume of waste: a large liquid waste stream is converted to a much smaller amount of solid waste, which can perhaps be more easily disposed of. In the second case, it is possible to avoid waste generation altogether and recover the metal.

The feasibility of metal recovery from biosorbent is generally governed by economic factors. For example, the price of the metals and the cost of the recovery process. Biosorbed heavy metals can be recovered from biosorbent either by destructive or non-destructive methods. Destructive methods may exterminate the biomass whereas non-destructive methods can reserve the biomass for future use by treating it with mild chemical reagents to desorb the loaded metals. For economical reasons, biosorbents are encouraged to regenerated and reused. Therefore, the development of recovery techniques may pay more attention to the non-destructive methods.

The metal is desorbed from the biomass by mild chemical reagents, yielding reusable biosorbent and a highly concentrated metal solution (of a concentration of at least 100 times that of the original solution). This metal-containing solution than can be treated by precipitation or electrodisslysis.

Many types of eluants can be used to desorb metals from the biomass and regenerate biosorbent as well [Aidor et al, 1995; Gadd and White, 1992; Kuyucak and Volesky, 1989b; Tsezos, 1984]. Desorption can be based on two general principles, which may also act in combination: certain desorbing agents such as acids or metal salts provide cations that compete with the bound metal ions for binding sites, thereby "pushing them away" from the sites. The effectiveness of desorption depends in this case on the binding strength of the cation added to the biosorbent. The alternative is to employ ligands which
complex the metal in solution (e.g., EDTA), such that the concentration of free metal in solution is lowered. In this way, the metal is "drawn" into the solution and thus the metal uptake is reversed.

Criteria for the choice of desorbing agents are as follows [Kuyucak and Volesky, 1989b]: (i) a high ratio of regenerated sorbent to eluant volume; (ii) a high metal concentration factor (after desorption and before adsorption); (iii) efficient desorption (a minimum of ca. 95% of the metal has to be desorbed); (iv) fast kinetics; (v) selectivity; (vi) no structural damage of sorbent material; (vii) high uptake of biosorbent in the next cycle; (viii) low cost; and (ix) environmentally benign. Some of these criteria are conflicting: if a smaller volume of eluant is used such that a high concentration factor is achieved (criteria i and ii), the efficiency may drop (criterion iii). Mineral acids (0.1 to 1 M) have proven to be efficient low cost desorption agents [Aidor, 1995]. Although higher concentrations of eluant yield higher efficiency (criterion iii and concentration factors [criterion ii]), extreme pH values can, for example, cause damage to the biosorbent (criterion vi) [Kuyucak and Volesky, 1989b].

Tsezos (1984) had chosen and tested six different eluants on the uranium uptake by *Rhizopiiis arrhizii*. These were sulfuric acid, nitric acid, hydrochloric acid, ammonium sulfate, sodium bicarbonate and sodium carbonate. The experimental data suggested that sodium carbonate/bicarbonate was the most effective desorption reagents among the others, probably because the carbonate radical was a strong complexing agent of uranium [Tsezos, 1984]. It could effect near complete uranium recovery and high uranium concentration factors. Furthermore, it caused the least damage to the biomass and thus allowed the biomass uranium uptake capacity following multiple biosorption-desorption cycles to remain nearly 90% of the original value. However, 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]. Although mineral acids like hydrochloric acid (HCl) and nitric acid (HNO₃) 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 (H₂SO₄) was not as effective as the above two acids in elution and it was also found to bring 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]. It has also 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].

In another case, a mixture of carbonate/bicarbonate solution was 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].

Copper bound to immobilized *Saccharomyces cerevisiae* and *Trichoderma viride* could be eluted by hypochloric acid (HCl₂₄) 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 rnmigera* [Kuhn and Pfister, 1989].

A method of selective metal recovery from algal biomass was demonstrated by Darnall et
al. (1986). 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 was 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.

In view of the economical and practical point, the desorption elution should be same to biosorbent regeneration reagent so that while heavy metal is desorbed from biosorbent, the regeneration of biosorbent is completed simultaneously. Chua et al (1998) demonstrated that recovery of bound Cu$^{2+}$ from *Pseudomonas sp.* cell biomass and regeneration of the biosorbent could be efficiently completed by diluted HCl simultaneously, and the regenerated *Pseudomonas sp* cell biomass could be reused effectively for removal and recovery Cu$^{2+}$ from wastewater at least five adsorption/desorption cycle.

### 2.4.7 Cell Immobilization Technique

All the existing or proposed industrial applications of biosorption for heavy metal removal use various form of immobilized biomass [Brierley et al., 1986; Krambeer, 1987]. Microbial biomass in its natural form consists of small particles of low density, low mechanical strength, and low rigidity. The use of such particles in any conventional unit operation for contacting the biomass with a large volume of solution containing metal ions has been shown to not be practical. The main difficulty lies in the rapid and efficient separation of the biomass from the reaction mixture after contact [Treen, 1981; Brierley et al., 1986; Mellis, 1986; Krambeer, 1987; Tsezos et al., 1987]. Alternatively, immobilized microbial biomass could be produced in the form of particles of desirable size, mechanical strength, and rigidity while maintaining the native properties of the
biomass. Thus, it can improve their performance in bioreactors as well as enhancing reusability [Volesky, 1987].

Many researchers [Brierley et al. 1986; Fukuda, 1995; Volesky, 1987] showed that immobilized microbial cells prevail over the freely suspended cells as they have the following advantages:

1. easy separation of cells from the reaction mixture;

2. better capability of reusing the biomass;

3. minimal clogging in continuous flow systems;

4. particle size can be controlled and high flow rates achieved with or without recirculation;

5. high biomass loading;

6. improved catalytic stability of immobilized cells;

7. microorganisms more inert to microbial contamination and biodegradation;

8. good mechanical stability;

9. provide resistance to shear for shear-sensitive cells;

10. operational stability is generally high.

However, the simultaneous achievement of all the above objectives is not an easy task. The biosorptive equilibrium properties of the microbial biomass can be protected to an extent during immobilization. The kinetic biosorptive characteristics of the native biomass are more difficult to protect.

For optimizing immobilized cell for efficient adsorption heavy metal in bioreactor, immobilization techniques should be extensively studied. The choice of proper support or
method will be governed by factors such as cost, ease of preparation, mechanical stability, biocompatibility and resistance against biodegradation. The principal techniques found in the literature for biomass immobilization are based on (1) adsorption on inert supports; (2) entrapment in a polymeric matrix; (3) covalent bonds to vector compounds; or (4) cells cross-linking [Veglio and Beolchini, 1997].

1. Adsorption on inert supports

Support materials are added into the tower fermenter before sterilization and inoculation with starter culture. They are left inside the continuous culture for a period of time. Then, a biofilm (a film of microbial cells) will be formed on the surface of supports [Veglio' and Beolchini, 1997]. Zhou and Kiff (1991) successfully used this technique for immobilization of Rhizopus arrhizus fungal biomass in reticulated foam biomass support particles. Scott and Karanjkar (1992) also used this for the formation of Enterobacter aerogenes biofilm attached to activated carbon.

2. Entrapment in polymeric matrices

The polymers commonly used to entrap microorganisms are, namely, calcium alginate, polyacrylamide, polyethyleneimine, polyhydroxyethylmethacrylate and polysulfone. The materials of calcium alginate and polyacrylamide used for immobilization are in the form of gel particles. Cells of Streptomyces viridochromogenes were immobilized by calcium alginate entrapment method to biosorb uranium from sea and fresh water [Nakajima et al., 1982]. Macaskie et al (1987b) used polyacrylaide gel-immobilized cells of Citrobacter sp. to remove cadmium.
3. Covalent bonds to vector compounds

The most common vector compound (carrier) is silica gel. The material is in the form of gel particles. This technique is usually found in the application for algae immobilization. Mahan and Holcombe (1992) successfully used this method to immobilize alga cells on silica gel for characterizing trace metal preconcentration.

4. Cross-linking

Stable cellular aggregates are formed with the addition of a cross-linker. The cross-linkers commonly employed are: formaldehyde, glutaric dialdehyde, divinylisureone and formaldehyde-urea mixtures [Holan et. al., 1993]. This technique was also used for immobilization of algae.

5. Magnetite Immobilised Cell

Recently, a novel magnetite immobilization technique seems to be the most attractive [Chua et. al., 1998; Sze et. al., 1996; Wong, 1993b]. The magnetite-immobilized bacterial cells have two distinct advantages: the separation of the immobilized cells from the treated metal-laden effluent is efficient and convenient, and the material properties of the biosorbent may be manipulated with immobilization methods. The macroscopic immobilized cells are more easily retained in a bioreactor operated in a continuous-flow mode, and pipeline blockage and filter clogging by the free-suspending microscopic microbial cells can be avoided.

Satisfactory immobilized biomass types, also called biosorbents, resemble a conventional exchange resin in physical form. The principal applications of immobilized biosorbents are in the sequestering of metal from wastewater in more rigorous industrial application
Most of the techniques for production of immobilized biomass (biosorbent) are proprietary.

2.4.8 Modelling the Biosorption Equilibrium

After establishing the general phenomena and trends in biosorption, the next step before applying it on an industrial scale is to develop mathematical models that describe the process quantitatively and aid in optimizing operation conditions. Even for bench scale experiments, mathematical modelling can assist in reducing the number of experiments performed: the initial conditions of the experiment can be chosen more judiciously if one knows what outcome may be expected. Instead of systematically trying all possible variations of all parameters, one can limit the experiments to a number of spot-checks under relevant conditions in order to verify the validity of the model. Modelling is all the more important for applications on an industrial scale since any trials at this level are rather expensive. Modelling can aid the reactor design and help discover bottlenecks and optimize the operating conditions.

In this section, equilibrium models are presented. The objective is to obtain models which are not just arbitrary statistical correlations but which are compatible with the observed binding mechanisms and which can be interpreted in a meaningful physicochemical way.

1. Models for adsorption isotherms

The adsorption of metal ions by the surface of biosorbent may be conceptually represented as the formation of a surface-metal complex:

\[ M + S = [MS] \]
where $M$ is free metal species,

$S$ is unoccupied surface site of biosorbent, and

$[MS] =$ surface-metal complex.

The biosorptive metal uptake can be quantitatively evaluated from experimental biosorption equilibrium isotherms. Upon contact between the biosorbent and the sorbate (metal) in solution, equilibrium is established at a given temperature. However, a certain amount of the sorbate sequestered by the biosorbent is in equilibrium with its residue left free species in the solution.

Giles et al. (1960) described "adsorption isotherm" as a plot of bound or adsorbed solute concentration ($q$) against the free solute concentration in solution (equilibrium concentration of solute) $Ce$ at a fixed temperature. Classifications systems divide all isotherms into four main types according to the initial slope and sub-groups classified based on the shape of the upper parts of the curves. They are S, L (Langmuir type), H (high affinity) and C (constant partition) isotherms as depicted in Figure 2.10
Figure 2.10 Four types of isotherms in classification system

equilibrium concentration of solute adsorbed (q)
equilibrium concentration of solute in solution (Ce)

H
L
C
S
Simple isotherm models such as Langmuir [Crist et al., 1992; Ferguson and Bubela, 1974; Kuyucak and Volesky, 1989a] and Freundlich [Chen et al., 1990; Tsezos and Deutschmann, 1990] sorption isotherms, which predict metal uptake as a function of the concentration of that one metal, are commonly used in biosorption. In their basic form, both models describe the binding of one sorbate; i.e., they are suited for mono-metal systems without pH effects. The Freundlich isotherm can be interpreted as sorption to sites with an affinity distribution whereby the sites with higher affinity for the metal become occupied first [Smith, 1981; Stumm, 1992]. The metal uptake, q (in milliequivalents per gram) is then given by

\[ q = k C_e^{1/n} \]

where \( k \) is related to the maximum binding capacity and \( n \) is related to the affinity or binding strength. It can be linearized by taking the natural logarithm on both sides of the equation:

\[ \ln q = \ln k + (1/n) \ln C_e \]

The intercept (\( \ln k \)) gives a measure of the adsorbent capacity when the solute concentration is low and the slope (\( 1/n \)) gives the intensity of adsorption.

Langmuir isotherm is derived from the dynamic equilibrium of the adsorbent and the adsorbate with several assumptions. The stated assumptions of the original Langmuir model are [Volesky and Prasetyo, 1994]:

1. The surface consists of adsorption sites;

2. All adsorption species interact only with a site and not with each other;

3. Adsorption is limited to a monolayer; and

4. Adsorption energy of all sites is identical and independent of the presence of adsorbed
species on neighbouring sites.

A general equation of Langmuir model is \( q = \frac{K C_0 q_{\text{max}}}{(1 + K C_0)} \)

The equilibrium constant \( K \) expresses the affinity between metal and biomass. \( q_{\text{max}} \) is maximum value of metal uptake, which is reached at high concentrations, corresponds to the total number of binding sites. The slope is proportional to \( K \). It is desirable that both a high metal binding capacity (\( q_{\text{max}} \)), and a high affinity (\( K \)) (i.e., a steep slope in the origin) occur.

Both the Langmuir and Freundlich isotherms have been applied successfully to model biosorption. Their drawback is, however, that these simple isotherms cannot predict the effect of pH or other ions in solution. In multi-solute solutions, which are encountered in most industrial applications, the ionic matrix of the solution is complex. The equilibrium of biosorption, as defined in single-solute solutions, may be affected significantly by the presence of other cations or anions in solution, depending on the chemical interaction of the other ionic species (co-ions) with the metal of interest and the biomass. Thus, although the biosorption of heavy metals is not affected significantly by the presence of alkali metals like sodium or potassium in solution [Polikarpov, 1966; Tsezos and Volesky, 1982], uptake of the uranium and radium by microbial biomass is affected strongly by the presence of heavy-metal ions such as zinc, copper, or iron in solution; the effect is not, however, the same for all biomass types [Tsezos and Volesky, 1982; Tsezos et al., 1986]. pH is another important parameter that influences the biosorption equilibrium, which affects the speciation of the ions in solution as well as the chemistry of the active sites on the biomass.

2.4.9 Reactor Types for Industrial Biosorption Processes
In practical, a better biosorbent associated with an appropriate bioreactor would further enhance the heavy metal biosorption efficiency. To design an appropriate bioreactor for biosorbent, the following factors should be considered [Fukuda, 1995]:

1. mass transfer limitations
2. viability maintenance
3. stabilization of immobilized cells

Several types of reactors could be used for biosorption, and each has certain advantages and drawbacks [Brierley, 1990; Brierley et al., 1986; Volesky, 1990b]. Stirred tank reactor, fixed packed bed reactor, pulsating beds and fluidized beds will be discussed briefly in the following section.

1. Stirred tank reactor (Figure 2.11)

Stirred tank reactor can be operated in batch or continuous modes. The biosorbent in contact with the metal-bearing liquid by maintaining it in suspension with stirring required for homogeneity and good mass transfer between the solid and liquid phases. If the biosorbent particles are fragile, such that they would be damaged by impellers, the tank can be air mixed. As the metal becomes sequestered by the biosorbent phase, the metal laden biomass is removed from suspension, for example, by gravity settling, flotation, centrifugation, filtration, or magnetic separation (if the biomass is magnetic) before the metal can be eluted to regenerate the biosorbent for recycling. Various multistep modifications of the basic continuous-flow stirred tank contact scheme can be developed. These multistep operations increase the efficiency as well as the operating costs of the process. Examples of the multistep biosorption designs are the countercurrent multistep arrangement and the multistep divided arrangement [Volesky, 1987]. The major
advantages of this reactor are operation ease, equipment maintaining simple, low capital cost, high feasibility and biosorbent could be regenerated and reused many times. The disadvantages are more biomass has to be used to achieve the same effluent quality as in the other techniques, and a solid/liquid separation step has to be needed.

Ginter and Groblicki (1997) applied a laboratory-scale stirred reactor for manganese uptake. The biosorbent was developed from solid sludge from anaerobically treated wastewaters. Magnetite-immobilized cells of *Pseudomonas* *sp* were used for removal and recovery of copper ions (Cu$^{2+}$) from electroplating effluent in a CSTR bioreactor [Sze *et al.*, 1996].
Figure 2.11 Schematic diagram of a biosorbent process based on a continuous-flow stirred tank reactor.
2. Fixed packed-bed reactor (Figure 2.12)

The most frequently used contactor is a packed-bed column, operating in a mode similar to the one where synthetic ion-exchange resins are employed.

The fixed packed-bed reactor is usually represented by a column arrangement where the biosorbent granules are packed into a solid bed, which does not normally move. The metal-bearing liquid percolates through the bed of active biosorbent. The biosorbent granules have to be large enough (1 to 3 mm) to avoid an excessive pressure drop across the bed. Too large a particle size would tend to decrease the effective surface area of the biosorbent, making the process possibly limited by the intraparticle diffusion rate [Volesky, 1990b]. As the layer of the reactor becomes saturated, the line of saturated biosorbent effectively progresses through the bed in the direction of flow. Consequently, all the biosorbent in the bed becomes saturated and inactive. This can be observed with a sharp increase in the concentration of metal in the effluent (the breakthrough point). The saturated column is then regenerated by passing through the smallest possible amount of appropriate desorption reagents in order to keep the metal in high concentration [Volesky, 1987].

The active duty can be shared by two or more columns; one is on the uptake cycle, and the other(s) is being regenerated or refilled. What's more, the columns can be operated in series for better control over the biosorbent performance or in parallel for increased removal capacity of the system.

The major advantage of choosing packed bed columns as reactor for biosorption is that it combines a high sorption capacity with achieving very low effluent concentration. There is no need to apply an additional solid-liquid separation step. Potential disadvantages are that the column could be clogged if the wastewater contains significant concentrations of
suspended solids, and the excessive channeling (i.e., axial mixing) may occur.

When the suspended foreign material becomes deposited on or in the upper part of the bed, functioning like an effective filter and causing high pressure drop of the column. For this reason, the column has to be either taken out of service or backwashed. The only effective remedy for this problem is a pretreatment removing the suspended solids from the influent.

Volesky and Prasetyo (1994) employed a packed-bed reactor for removing cadmium by a brown marine alga Ascophyllum nodosum. Multiple fixed-bed columns packed with calcium alginate (CA)-immobilized biomass of Pseudomonas aeruginosa PU21 were utilized to remove lead, copper and cadmium from the contaminated water [Chang and Huang, 1998].
Figure 2.12 Schematic diagram of a biosorbent process based on a fixed-bed column reactor

Metal-loading influent

Possibly Recirculation

Effluent
3. Fluidized bed contactor (Figure 2.13)

In fluidized or expanded-bed reactors, the wastewater passes upward through the reactor. The flow rate has to be balanced with the biomass size and density such that the flow suspends the biomass particles without carrying them out of the reactor. Due to its fluidized movement, the biosorbent bed often occupies a larger volume and space in the column when compared with a fixed bed system. Similarly to fixed-bed columns, the water leaving the reactor is in contact with relatively fresh biomass. However, the metal concentration profile in fluidized-bed reactors is more blurred than in fixed-bed columns. Fluidized-bed reactors avoid the danger of clogging. The metal-loaded particles tend to exhibit a higher density, so that they sink to the bottom of the reactor, where they can be periodically collected and replaced with fresh biosorbent (about 1/10 of the biomass in the reactor is replaced at a time). The major disadvantage of fluidized-bed system is to operation difficulty, and has a tendency to lose a portion of active biosorbent due to it upflow direction operation.
Figure 2.13 Schematic diagram of a biosorbent process based on a fluidized-bed reactor
4. Pulsating bed contactor (Figure 2.14)

In this process, the metal solution is usually operated in upward flow direction. The "end" of the bed is replenished with a batch of a fresh or regenerated biosorbent material. The saturated laden biosorbents are periodically replaced and withdrawn from the bottom of the column in a counter-current fashion. As a result, continuous operation is permitted. However, the metal laden biosorbents have to be regenerated separately [Volesky, 1987].
Figure 2.14 Schematic diagram of a biosorbent process based on pulsating tank reactor

(regenerated) BIOSORBENT

PULSE TANKS

TREATED EFFLUENT

REACTOR

METAL-BEARING SOLUTION

REGENERATION
2.4.10 Advantages of Biosorption for Removing Heavy Metal from Industrial Waste

Effluent

Conventional methods employed for removing metals from industrial wastewater include chemical precipitation, chemical oxidation or reduction, filtration, electrochemical treatment, membrane technology and evaporation recovery. However, many of these procedures have significant disadvantages, namely, high reagent requirements, generation of toxic sludge or are very expensive when the contaminant concentrations are less than 100 mg L\(^{-1}\) [Sag and Kutsal, 1996].

Comparison with conventional methods, biosorption technology for removing heavy metal from industrial waste effluent have the following advantages:

1. Their heavy metals removal capacities are apparently higher than those of conventional methods. For example, the uptake of uranium by dead fungal mycelia, Rhizopis arrhizus, has been reported with removal capacity 180 mg U per gram of the sorbent, which was 10-20 times higher than those of commercially available ion exchange resins [Tsezos and Volesky, 1981].

2. Not only the metal uptake capacity of biomass is high, the uptake can also be metal selective [Tobin et al., 1984; Treen-Sears et al., 1984c]. Usually, the metal of interest was found in a mixture with other metals that may not be required during recovery. Thus, the selectivity of the overall biosorbent process is very important.

3. The biosorbent used may be more economical. Biosorbents might consist of raw biomass; which can be supplied either as waste materials from industrial processes, for example, industrial fermentations and biological wastewater treatment, or as naturally abundant renewable biomaterial like algae and fungi [Volesky, 1987]. It is also possible to propagate the microbial biomass in wastes from various industries. Therefore the
biosorbents used could be very economical for detoxifying industrial wastewater solutions.

4 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\(^{-1}\)) and the effluent must contain less than 1 mg L\(^{-1}\) heavy metals [Tsezos and Keller, 1983; Ross, 1989; Volesky, 1987].

2.4.11 Limitation and Further Studying for Employing Biosorption to Remove and Recovery Heavy Metal from Industrial Effluent

From the practical operation point of view, biosorbent regeneration, disposal and transport contributed to major cost of biosorption process comparing with biosorbent production [Atkinson et al., 1998]. For reducing use amount of biosorbent, thus minimizing the biosorbent regeneration, disposal and transport cost, the metal adsorption capacity of biosorbent should be enhanced as far as possible.

A "rule of thumb" for the economic threshold for commercial selection of a biological process to replace a physico-chemical process for heavy metal removal from a waste stream is that a metal loading capacity greater than 15% of the biomass dry wt. must be realised [Macaskie, 1991].

Before the selection of any technology, it is imperative to note the hazardous waste management options: Reduce. Reuse. Recycle. The option of last resort is to treat and dispose of the waste in safe landfills, while minimizing the resultant volume, since disposal sites are few and space is precious.

A given biosorption technology should be able to perform on a large scale in order for it to be commercially viable. The organism or biomaterial selected to accomplish the goal
of removing metals from the environment must be very efficient in performing its intended function. The literature abounds with reports of studies attesting to the "potential" of a particular biomass or biomaterial to carry out metals biosorption, but few have actually ventured beyond the laboratory scale.

For biosorption to become competitive with existing technology, many aspects should be further considered and studied. For example, (1) cell adsorption capacity to heavy metal should be improved as far as possible to reduce the using amount of biosorbent and thus minimizing the sludge regeneration, disposal and transport cost; (2) metal adsorption and desorption properties of the biosorbent should be further clarified for optimizing biosorption operation; (3) process design should be considered to provide comfortable full scale bioreactor with biosorbent for efficient heavy metal biosorption.

For improving heavy metal binding capacity of biosorbent, efficient screening technology and optimal biosorbent production technology should be further studied. Genetic engineering technology can be introduced to modify the structure and components of cell surface to further enhance the metal binding capacity. There were many literatures showed that bacterial cell biomass has different metal binding capacity in different growth age due to the change of cell surface structure and components. PEGs, proteins, phospholipids and lipopolysaccharides were considered as major metal binding active components. However, which component variation in different growth age mainly result in the diverse of heavy metal binding capacity was apparently neglected in the literatures, such information can serve as an useful aid for enhancing metal adsorption capacity of bacterial cell through modifying cell surface structure and composition by genetics engineering. Other technologies can be used to entrap or encapsulate the isolated proteins into gels where they can function in an in vitro manner if desirable. Metal-binding proteins could trap metal ions in a more efficient manner and perhaps be amenable to the
reverse reaction and provide a reusable system.
Chapter 3: Materials and Methods

3.1 Screening method for bacteria with high heavy metal binding capacity

A rapid plate screening method was developed to select high heavy metal accumulating bacteria from contaminated site as biosorbent for adsorption and recovery of Cu$^{2+}$ and Ni$^{2+}$ from electroplating effluent. The operation procedure was detailed below:

3.1.1 Chemical

Heavy metal salt: stock solution containing 26.85 g L$^{-1}$ of CuCl$_2 \cdot 2$H$_2$O (10000 mg Cu$^{2+}$ L$^{-1}$) and 25.44 g L$^{-1}$ NiCl$_2$ (10000 mg Ni$^{2+}$ L$^{-1}$), respectively, 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.

Metal ion precipitation reagent: 2% (w/v) (NH$_4$)$_2$S. 2g (NH$_4$)$_2$S was dissolved in 100 ml distilled water.

3.1.2 Media

The screening medium (SM) was modified from Kwok (1990): D-glucose, 4.0 g; NH$_4$Cl, 1.02 g; K$_2$HPO$_4$, 0.057 g; NaCl, 1.0 g; K$_2$SO$_4$, 1.0 g; MgSO$_4 \cdot 7$H$_2$O, 0.05 g; CaCl$_2 \cdot 2$H$_2$O, 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$_4 \cdot 7$H$_2$O and CaCl$_2 \cdot 2$H$_2$O 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 SM and semi-solid SM medium was made by adding 15 g and 8 g of agar, respectively. For Cu²⁺ or Ni²⁺ containing semi-solid SM medium, separately autoclaved stock solution of CuCl₂·2H₂O or NiCl₂ were added by appropriate volume to attain a final concentration of exception.

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.

3.1.3 Isolation of Bacteria from Heavy Metal Contaminated Side

0.1 ml wastewater sample from heavy metal contaminated side were added to sterile saline solution (NaCl 0.85 %) and serially diluted to 10⁻⁵ with the solution. 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 %).

3.1.4 Selection of Cu²⁺ Resistant Bacteria from Wastewater Samples

Wastewater samples collected from Fo Tan Nullah were added to test-tubes containing 5 ml of SM with Cu²⁺ at final concentration of 100 mg L⁻¹. The test-tubes were incubated at 30 °C and agitated at 200 rpm until microbial growth was observed. 0.1 ml of the culture were taken out, serially diluted, plated on LB agar containing 100 mg Cu²⁺ L⁻¹ and incubated at 30°C overnight. Colonies of distinct morphologies were picked and inoculated again to test-tubes of SM with 100 mg L⁻¹ Cu²⁺ to check their Cu²⁺ resistance. For those tubes showing cell growth, 0.1 ml culture were taken out, serially diluted and
plated on LB agar so as to confirm purity of culture by observing colony morphologies.

3.1.5 Pre-screening of Bacteria for Copper Uptake

Pre-screening method was modified from the method of Thomas (1995). Bacteria isolated from heavy metal contaminated wastewater, Cu\(^{2+}\) resistant bacteria selected from wastewater sampling from Fo Tan Nullah were inoculated onto SM agar plates. After incubation at 30 °C overnight, agar plates with well-developed colonies were overlayed with a semi-solid SM containing 20 mg L\(^{-1}\) Cu\(^{2+}\). After further incubation 12 hours, freshly prepared 2% (NH\(_4\))\(_2\)S was sprayed on plate surface. Visual black haloes could be found in the surrounding area of some colonies. This is because the colonies accumulated Cu\(^{2+}\) in its surround area so that produce large amount of CuS precipitation after spraying (NH\(_4\))\(_2\)S. Thus the colonies were tentatively considered to possess high Cu\(^{2+}\) accumulating capacity, and were picked out for further screening procedures.

3.1.6 Determination of Copper Binding Capacity of Selected Bacteria

1. Culture of Bacteria

Bacterial inoculum was prepared by inoculating single colony to SM in test-tubes. It was incubated at 32 °C and agitated at 200 rpm for 24 hours to serve as inoculum. 250 ml flask containing 100 ml SM was added with 1 % inoculum, incubated at 32 °C and agitated at 200 rpm for 36 hours. The cells were then harvested by centrifugation (4,000 g; 15 min; 4 °C), washed twice with TRIS buffer and then resuspended in 10 ml of the buffer.

2. Determination of Copper Binding Capacity
10ml of the bacterial cell suspension was added into a centrifuge bottle containing 90 ml of TRIS buffer with 50 mg L$^{-1}$ of Cu$^{2+}$. For the control experiment, 10 ml of TRIS buffer was added. After equilibration by shaking (200 rpm, 30 °C, 1 hour), the bacterial cells were removed by centrifugation (8000 g, 15 min, 20 °C). The supernatant was decanted into a polyethylene bottle for determination of Cu$^{2+}$ concentration. The dry weight of cells added was deduced by taking 10 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 10 ml of TRIS buffer was also determined.

3. Determination of Cu$^{2+}$ Concentration

Cu$^{2+}$ concentration was determined by Perkin Elmer 3300 atomic absorption spectrophotometer.

4. Calculation of Cu$^{2+}$ Binding Capacity

Binding capacity (RC) of Cu$^{2+}$ expressed as mg Cu$^{2+}$ removed per g cell dry weight and calculated by the following equation:

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

where $C_c$ is the final Cu$^{2+}$ concentration in the control (mg L$^{-1}$),

$C_e$ is the residual Cu$^{2+}$ concentration after addition of the cells (mg L$^{-1}$),

$W$ is the cell dry weight (g), and

$V$ is volume of the Cu$^{2+}$-containing buffer solution (L).

The bacterial isolate with the highest Cu$^{2+}$ binding capacity was selected for further
studies.

3.1.7 Screening of Ni²⁺ Binding Bacteria from Cu²⁺ Binding Bacteria

Selected bacteria with high Cu²⁺ binding capacity were inoculated onto SM agar plates. After incubation at 30 °C overnight, agar plates with well-developed colonies were overlayed with a semi-solid SM containing 20 mg L⁻¹ Ni²⁺. After further incubation 12 hours, freshly prepared 2% (NH₄)₂S was sprayed on plate surface. The colonies with visual black haloes in surrounding area due to the formation of NiS precipitation were tentatively considered to also possess high Ni²⁺ accumulating capacity, and were picked out for further screening procedures.

3.1.8 Determination of Ni²⁺ Binding Capacity of Selected Bacteria

The test procedure is same as to determination of Cu²⁺ capacity in section 3.1.6.

3.2 Identification of Bacteria

Bacteria identification was carried by Department of Biology, Chinese University of Hong Kong based on the classification scheme in Bergey's Manual [Holt, 1994].

3.3 Cell Culture and Biosorbent Preparation

3.3.1 Chemicals Reagent

2-(N-morpholino)ethanesulfonic acid (MES) buffer: a buffer solution containing 10 mM MES with pH adjusted to 6.0 by potassium hydroxide was used in the following
experiments. This MES buffer solution might be added CuCl₂ to contain 50 mg L⁻¹ Cu²⁺ and NiCl₂ to contain 50 mg L⁻¹ Ni²⁺, respectively, and was designated as Cu²⁺ containing buffer and Ni containing buffer in the following text.

3.3.2 Media

Sulfate Limiting Medium

D-glucose, 10.0 g; NH₄Cl, 1.5 g; K₂HPO₄, 1.5 g; NaCl, 1.0 g; KCl, 1.0 g; MgCl₂·6H₂O, 0.2 g; CaCl₂·2H₂O, 0.02 g; FeSO₄·7H₂O, 0.002 g; TRIS, 7.5 g and Yeast Extract, 0.1 g dissolved in 1 litre of distilled water. Medium's pH was adjusted to 7.5 with concentrated HCl.

3.3.3 Cell Culture

The biosorbent used in this study was *Pseudomonas putida* 5-x cell, which was isolated from the local electroplating industrial effluents. The microbes were cultured in Luria-Bertani medium (LB) and then mixed with glycerol (final concentration 25%) and stored at -70°C. Bacterial inoculum was prepared by inoculating a single colony in 5 ml sulphate-limiting medium (SLM) in a test-tube and incubated at 30°C with shaking at 200 rpm for 24 hours [Wong et al, 1993b]. The SLM, contained 1% inoculum, was then incubated in flask at 30°C with shaking at 200 rpm for 48 hours [or in a 16-L fermentor (Bioengineering Co.) at an agitation rate of 400 rpm and an aeration rate of 4.0 L min⁻¹ at 30°C for 36-48 hours]. The microbes were harvested by centrifugation at 12000 rpm for 10 min at 4°C. The cell pellets were washed twice using 100 ml 2-(N-morpholino)ethanesulfonic acid (MES) buffer [So, 1991] and then collected by centrifugation for cell
pretreatment and immobilization

3.3.4 Cell Pretreatment

Collected cells harvested under optimal growth phase were resuspended in different pretreating solution, such as HCl, NaOH, EDTA, for 30 minutes, or heat treated by autoclaving for 10 min (121 °C; 15p). Samples of pretreated cells were observed under light microscope. The cell treated by various pre-treatments was washed twice with MES buffer and then was immobilized.

3.3.5 Preparation of magnetite - immobilized cells of *Pseudomonas putida S-x*

The pretreated cells were collected by centrifugation and were then resuspended in the MES buffer solution. Then magnetite (Aldrich, USA) with 5:1 ratio of magnetite weight to cell dry weight was added into the suspension. The mixture was stirred at 200 rpm for 30 min at 25°C, and then the mixture was settled for 10 min with the aid of an electromagnet. More than 99.0 % of bacterial cells could be immobilized on magnetite (Microscope test).

The schema of cell culture and cell pretreatment and immobilization was showed in Figure 3.1.
Figure 3.1 Cell culture and biosorbent preparation
3.4 Biosorption Studies by *P. putida* 5-x Cell Biomass

3.4.1 Adsorption Process and Adsorption Capacity

Batch experiments for determining metal biosorption properties were carried out with the initial Cu\(^{2+}\) or Ni\(^{2+}\) concentrations ranging from 5 to 100 mg L\(^{-1}\) and cell biomass concentrations at 0.2~1.0 g dry cell L\(^{-1}\). The cell biomass was added into a 200 ml beaker containing 100 ml pH 6.0 MSB buffer solution containing Cu\(^{2+}\) and Ni\(^{2+}\), respectively. All experiments were performed in duplicate. Control experiments were carried out under identical conditions, but without any biomass. The biosorption process was carried by agitation with magnetic stirrer at 200 rpm at 25\(^\circ\)C for 60 min, at intervals, samples were taken and the biomass was separated using centrifuge. The concentrations of Cu\(^{2+}\) or Ni\(^{2+}\) in the supernatants were determined using the atomic absorption spectrophotometer. Cell dry weight was determined by the same procedure as section 3.1.6

The Cu\(^{2+}\) and Ni\(^{2+}\) adsorption capacity was expressed as following equations:

\[
Q = (C_c - C_e) V / W
\]

where \(C_c\) is final Cu\(^{2+}\) or Ni\(^{2+}\) concentration in the control (mg L\(^{-1}\)),

\(C_e\) is residual Cu\(^{2+}\) or Ni\(^{2+}\) concentration in supernatant after biosorption (mg L\(^{-1}\)),

\(W\) is cell dry weight (g),

\(V\) is volume of the Cu\(^{2+}\) or Ni\(^{2+}\)-containing buffer solution (L).

3.4.2 Kinetics of Copper and Nickle Uptake

Cells cultured under optimal conditions were added into a plastic bottle with 500 ml of Cu\(^{2+}\) or Ni\(^{2+}\) containing buffer solution. The solution was mixed by a magnetic stirrer at 25\(^\circ\)C. Samples of the cell suspension were taken out at 2 min interval and removed
biomass by centrifuge. The Cu$^{2+}$ and Ni$^{2+}$ concentrations of the samples and the removal capacities were determined as described in section 3.4.1, and the types of adsorption kinetics could be determined using concentration of Cu$^{2+}$ (or Ni$^{2+}$) in supernatant plotted against the adsorption times.

3.4.3 Effect of Cell Metabolism on Copper Adsorption

Metabolism inhibited cells were prepared by pretreatment with 5 mM NaN solution for 1 hour. The cells were then washed twice with MES buffer and Cu$^{2+}$ removal capacity was determined as described in section 3.4.1. Effect of glucose was investigated by suspending the cells in the Cu$^{2+}$ containing buffer solution in the presence of 0.5 % glucose under conditions described in section 3.4.1. Cu$^{2+}$ removal capacity was determined as above.

3.4.4 Adsorption Isotherm for Copper and Nickel Uptake

Different biomass, such as intact cells, envelopes and separated cell components, was added into the buffer solutions with initial Cu$^{2+}$ concentrations of 5, 10, 25 and 50 mg L$^{-1}$ (or Ni$^{2+}$ concentration of 4.6, 9.2, 23.1, 36.5 and 46 mg L$^{-1}$). The Cu$^{2+}$ and Ni$^{2+}$ binding capacities were determined as described before, respectively. According to the results of adsorption capacity and equilibrium concentration of Cu$^{2+}$ (or Ni$^{2+}$) in solution, the adsorption isotherm could be determined using the Cu$^{2+}$ (or Ni$^{2+}$) adsorption capacity plotted against the equilibrium concentration of Cu$^{2+}$ (or Ni$^{2+}$) remained in supernatant.

3.4.5 Effect of Cell Culture Conditions on Copper and Nickel Adsorption Capacity
1. Effect of Nutrients 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 3.1. LB broth added with 7.5 g L⁻¹ TRIS was also used. All media’s pHs were adjusted to 7.5 with concentrated HCl. No pH change in the media was observed after autoclaving.

Culture of cells

Bacterial cells were incubated in the 4 media shown in Table 3.1 and LB broth 500ml-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 (4000 g; 10 min; 4 °C), washed twice with MES buffer and then resuspended in 10 ml of the buffer solution.

Determination of Copper and Nickle Adsorption Capacity

Cu²⁺ and Ni²⁺ adsorption capacity of the bacterial cells cultured in different media was determined as described in section 3.4.1.
Table 3.1 Composition in phosphate, ammonia, sulfate, glucose limiting media and nutrient non-limiting media for *Pseudomonas putida* 5-x

<table>
<thead>
<tr>
<th>Composition (g L⁻¹)</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>phosphate limiting</td>
</tr>
<tr>
<td>Tris</td>
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</tr>
<tr>
<td>NH₄Cl</td>
<td>1.5</td>
</tr>
<tr>
<td>K₂HPO₄</td>
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</tr>
<tr>
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</tr>
<tr>
<td>K₂SO₄</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>MgCl₂.6H₂O</td>
<td>0</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
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</tr>
<tr>
<td>FeSO₄.7H₂O</td>
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<tr>
<td>D-Glucose</td>
<td>10</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.1</td>
</tr>
</tbody>
</table>
2 Effect of Incubation Temperature and Growth Age on Copper and Nickle Adsorption

Medium

The sulfate limiting medium (SLM) described in Table 3.1 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). 500 ml flask containing 200 ml SLM were added with 2 ml inocula, incubated at 30, 34 or 37°C and shaken at 200 rpm. For each incubation temperature, cells were harvested after incubating 12, 24, 36 and 48 hours. Cells were harvested, washed and suspended in MES buffer as above described.

Determination of Copper and Nickle Adsorption Capacity

Adsorption capacity of the cells cultured in different condition was determined as describe in section 3.4.1

3.4.6 Determination of Optimal Adsorption Condition

1. Effects of pH on Copper and Nickle Binding Capacity

Cells of Pseudomonas putida 5-x cultured under optimal conditions were collected and suspended in 10 mM MSB solution. 10 ml of the cell suspension was added to Cu\(^2+\) containing solutions (or Ni\(^2+\) containing solution) 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\(^{2+}\) (or Ni\(^{2+}\)) containing buffer solutions after Cu\(^{2+}\) (Ni\(^{2+}\)) removal was measured. Cu\(^{2+}\) removal capacities at the different pHs were determined as described before.

2. Effects of Other Metal Ions on Copper and Nickel Binding Capacity

Cells cultured under optimal conditions were added to Cu\(^{2+}\) containing buffer solutions (or Ni\(^{2+}\) containing solution) with equi-molar concentrations (0.78 mM) of other metal ions in the form of chloride salts. Buffer solution containing only 50 mg L\(^{-1}\) Cu\(^{2+}\) (or 46 mg L\(^{-1}\) Ni\(^{2+}\)) was also used for comparison. Cu\(^{2+}\) (or Ni\(^{2+}\)) removal capacities of the cells in the presence of various metallic ions were determined as described before.

3. Effects of Interaction between Copper and Nickel on Adsorption of P. putida 5-x Cell Biomass

The simultaneous adsorption of Cu\(^{2+}\) and Ni\(^{2+}\) was studied in wastewater containing both Cu\(^{2+}\) and Ni\(^{2+}\). A set of adsorption experiments was carried out in wastewater containing both Cu\(^{2+}\) and Ni\(^{2+}\) with different ratio of molar concentrations such as 5:1, 2:1, 1:1, 0.6:1, 0.4:1, 0.2:1, 0.1:1 and 0.05:1, and compared with in wastewater only containing Ni\(^{2+}\) or Cu\(^{2+}\), respectively. The adsorption process and determination of Cu\(^{2+}\) and Ni\(^{2+}\) adsorption capacity was same to procedure of section 3.4.1

4. Effects of Anions on Copper and Nickel Binding Capacity

Cells were added to Cu\(^{2+}\) containing solutions (or Ni\(^{2+}\) containing solution) with 0.78 mM of boric acid (H\(_3\)BO\(_3\)), potassium carbonate (K\(_2\)CO\(_3\)), potassium sulfate (K\(_2\)SO\(_4\)) or 1.56
mM of potassium chloride (KCl). A buffer solution containing only 50 mg L⁻¹ Cu²⁺ (or 46 mg L⁻¹ Ni²⁺) was used in the control experiment. Cu²⁺ (or Ni²⁺) removal capacities in the presence of various anions were determined as described before.

5. Effects of Cell Pre-treatment on Copper and Nickle Binding Capacity

Cells cultured under optimal conditions were harvested and suspended in different eluant, such as HCl, NaOH, EDTA, for 30 minutes. Samples of pretreated cells were observed under light microscope. Cells treated by various pretreatments were washed twice with MES buffer and determined the Cu²⁺ or Ni²⁺ adsorption capacity as same procedure as above.

3.5 Desorption and Biosorbent Regeneration and Reuse

3.5.1 Bound Heavy Metal Desorption

After biosorption, the heavy metal bound on biosorbent was recovered using desorption eluant. Heavy metal bound biomass was recovered by centrifugation at 8000 rpm and 4 °C for 20 minutes. The biomass was washed with DDI water, centrifuged and resuspended in different desorption eluent and shaken at 25 °C and 250 rpm. After 1 hour, biomass was centrifuged, dried and weighed for reuse. Supernatants were collected for determination of Cu²⁺ (or Ni²⁺) concentration, and desorption efficiency.

3.5.2 Effect of Desorbing Solution Volume on Heavy Metal Desorption

1 g of Cu²⁺ or Ni²⁺ bound dry cell biomass was resuspended in 5, 10, 20 and 30 ml 0.05 M HCl or other desorbing solution for desorbing one hour. Finally, the biosorbent was
removed by centrifuge, and the Cu\(^{2+}\) (or Ni\(^{2+}\)) concentrations in the supernatants were analyzed and the desorption efficiency was determined.

3.5. 3 Kinetics of Desorption

Cu\(^{2+}\) (or Ni\(^{2+}\)) loaded biomass was harvested after biosorption. The biomass was rinsed with DDI water and then resuspended in 0.05 M HCl solution. The suspension was then agitated on a shaker at 250 rpm. Samples were taken from the suspension at designated time intervals. The samples were centrifuged immediately at 12,000 rpm to separate the biomass from the liquid. Cu\(^{2+}\) (or Ni\(^{2+}\)) concentrations in the supernatants were determined using the atomic absorption spectrophotometer, and the types of desorption kinetics were then determined using the Cu\(^{2+}\) concentration in supernatant plotted against the reaction times.

3.5.4 Regeneration and Reuse of Biosorbent (adsorption/ desorption recycles)

In the view of economical and practical point, the biosorbent regeneration reagent should be same to desorption solution so that while heavy metal is desorbed from biosorbent, the regeneration of biosorbent is completed simultaneously. Therefore the biosorbent regenerations were carried by various desorbing solutions which are efficient for Cu\(^{2+}\) and Ni\(^{2+}\) desorption. The reuse efficiency of regenerated biosorbent for Cu\(^{2+}\) and Ni\(^{2+}\) adsorption was assessed.

After biosorption, the Cu\(^{2+}\) (or Ni\(^{2+}\)) loaded biomass was centrifuged, rinsed with DDI water and resuspended in various desorbing/regenerating solutions for 60 minutes in order to desorbing Cu\(^{2+}\) (or Ni\(^{2+}\)) and regenerating biosorbent, simultaneously. The regenerated biomass was washed twice with 10 mM pH 6.0 MSB, and then suspended in
heavy metal containing solution again for the next biosorption run. These biosorption and desorption steps were repeated five times. The Cu^{2+} (or Ni^{2+}) concentrations in the supernatants were analyzed after each biosorption/desorption (regeneration) cycle for assessing adsorption efficient by regenerated biomass.

3.6 Removal and Recovery of Cu^{2+} or Ni^{2+} from Wastewater by Semi-Continuous Stirred Reactor System

A semi-continuous series stirred reactor system was used to remove and recover Cu^{2+} or Ni^{2+}. The biosorption system comprised a series of three batch biosorption reactors (R1, R2 and R3) as shown in Figure 3.2. Each reactor consisted of a 500-mL glass beaker, a magnetic stirrer and an electromagnet. The Cu^{2+} (or Ni^{2+}) bearing wastewater was fed counter-currently to the biosorbent (immobilized *P. putida* 5-x cell biomass) into the reactor in order to obtain the lowest possible Cu^{2+} (or Ni^{2+}) concentration in effluent and highest possible Cu^{2+} (or Ni^{2+}) concentration in the biosorbent. In adsorption/desorption process, the heavy metal containing wastewater was well stirredly reacted with biosorbent in each reactor for 30 min, and settling 20 min with aid of electromagnet. The supernatant of R1 then flows into R2 for second step adsorption, and then to R3 for third step adsorption. The supernatant of R3 finally was discharged with meeting discharge standard of Hong Kong. After adsorption and settling, the Cu^{2+} bound biosorbents in R3 and R2 were transformed to R2 and R1, respectively, for further adsorbing Cu^{2+} until to saturation. The saturated biosorbent by Cu^{2+} in R1 was passed through a 0.4 μ filter and then Cu^{2+} was recovered, and the biosorbent was regenerated in acid regeneration reactor. Regenerated biosorbent was returned into R3 for reuse after being washed by MES buffer in washing tank. Cu^{2+} adsorption capacity of magnetite-immobilized cell was determined by measuring the concentrations of Cu^{2+} in the
supernatant after settling (Using Perkin Elmer 3300 atomic adsorption spectrophotometer).

**Figure 3.2** Schema of treating Cu\(^{2+}\) bearing wastewater by magnetite-immobilized *P. putida*5-x

**R**: Biosorption Reactor; **F**: Filter; **DR**: Desorption and Regeneration Tank; **W**: Wash Tank
3.7 Removal of Copper and Nickel from Electroplating Effluent by Linked Semi-Continuous Stirred Reactor Using Immobilized *P. putida* 5-x Cells as Biosorbent

A sample of electroplating effluent containing large amount of Cu$^{2+}$ and Ni$^{2+}$ was treated by semi-continuous stirred reactor using immobilized *P. putida* 5-x cell as biosorbent (Figure 3.2). Due to the interaction of Cu$^{2+}$ and Ni$^{2+}$ in wastewater on adsorption of biomass, two sets semi-continuous stirred reactor system were linked serially to efficiently remove and recover Cu$^{2+}$ and Ni$^{2+}$ sequentially from electroplating wastewater. In first set, the immobilized cell biomass prepared as optimal biosorbent for Cu$^{2+}$ adsorption removed and recovered Cu$^{2+}$ from wastewater as same procedure as section 3.6. The effluent of the set, in which the Cu$^{2+}$ concentration was reduced to 2 mg L$^{-1}$ (0.03 mmol L$^{-1}$) or below, then flowed to second set for Ni$^{2+}$ removal. The immobilized *P. putida* 5-x cell biomass prepared as optimal Ni$^{2+}$ biosorbent in second set could efficiently removed and recovered Ni$^{2+}$ remained in effluent of set one. The respective Cu$^{2+}$ and Ni$^{2+}$ desorption and biosorbent regeneration and reuse were carried out as same procedure as section 3.6.

3.8 Study on the Adsorption Mechanism of *P. putida* 5-x Cell

Biosorption mechanisms, such as adsorption site and active group on cell surface, contribution of cell surface components and chemical ingredients to heavy metal adsorption, were investigated by using biochemical separation technology, scanning electron microscopy (SEM) and transmission electron microscopy (TEM), X-ray energy dispersion analysis, (EDAX) and cell surface electro-charge analysis technology.
3.8.1 Analysis under Scanning Electron Microscopy

Cells, cell envelopes and separated cell components, such as outer membrane, PEG of \( P.\ putida \) 5-x, before exposure to heavy metal solution, were spread out on some thin sheets of glass slides. The slides were then dried in an oven at 45 °C for 6 hours. Following that, the slides were fixed on aluminium stub and placed into a sample chamber for one night. The chamber was flushed full of argon gas under 0.02 to 0.04 mbar. Then, the samples were coated with a thin layer of gold by polaron SC502 Sputter Coater in order to avoid accumulation of electrons on the samples' surfaces. Finally, the specimens were examined under a scanning electron microscope (Stereoscan 440, Leica) with 15 kV accelerating voltage, 400 Pa probe current and 10 mm working distance.

3.8.2 Analysis under Transmission Electron Microscopy

Cu\(^{2+}\) bound \( P.\ putida \) 5-x cells harvested in different growth phase were examined as whole mount by a JEM-1200 EX-II TEM (JEOL Ltd., Tokyo, Japan) which was operated at 80 kV under standard conditions. For whole mounts, carbon-Formvar coated nylon grids (200 mesh) (Pelco International, Redding, CA, USA) were floated in aqueous cell suspensions for 10 s, dried with blotting paper and then examined. The surface structure of fresh cell, pre-treated cell was examined as thin sections. For thin sections, the sample pellets were harvested and concentrated by centrifugation, fixed with 2.5% glutaraldehyde and dehydrated by acetone. Then the pellets were embedded in Spurr at 68°C for 16h. Thin sections of 60 nm were prepared using a Reichert Ultracuts (Leica, Wien, Austria) equipped with a diamond knife (Diatome 45°, Fort Washington, USA). Sections were collected and examined under transmission electron microscope.
3.8.3 Analysis under X-ray Energy Dispersion (EDAX)

Samples of biomass were mounted at the aluminum stub by double-sided tape. The stubs were then fixed in the sample holder and dried in oven at 45 °C for 6 hours. After that, they were placed into the sample chamber of a scanning electron microscope equipped with a X-ray analyzer which was then flushed full of argon gas under 0.02 to 0.04 mbar. Finally, the EDAX spectra were recorded with a 15 kV accelerating voltage, 20 Pa probe current and 25 mm working distance.

3.8.4 Removal of Cell Superficial Layer—Capsule

Cell superficial layer-capsule could be removed easily by weaker eluant, [Geesey and Jang 1989]. For removing cell superficial layer, 100mg (dry weight) P. putida 5-x cell biomass were washed with 25 ml of 0.1 mol L⁻¹ HCl by shaking at 200 rpm for 20 min at 25°C. The washed cells were harvested by centrifugation at 4000 rpm for 20 min, and then washed twice with 50 ml of 10 mM MES buffer with pH 6.0. The surface structure of the superficial layer-capsule removed cell was observed and compared with fresh cell by TEM.

3.8.5 Preparation of Cell Envelopes

Bacterial cells were centrifuged at 6000 g at 4°C for 30 min, and the supernatants were removed. The cells were washed three times in a sterile saline solution to remove the maximum of loosely adsorbed material from the growth medium by centrifugation at 10000 g at 4°C for 30 min. Then the cells were suspended in pH 7.0 TRIS-HCl buffer and broken by pass twice through a French Pressure at 1400 kg cm⁻¹. The disintegrated cell suspensions were centrifuged at 3000 rpm for 10 min to remove remained whole cells. The supernatant was then treated with 10 mg L⁻¹ of deoxyribonuclease and 15 mg
L^{-1} of ribonuclease for 45 min at 25°C, then the envelopes pellets were separated from supernatant by centrifugation at 15,000 rpm for 60 min [Beveridge, 1982]. The harvested envelopes were washed five times with 0.05 molL^{-1} pH 7.2 HEPES (N-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid) buffer solution, and then washed twice with distilled water. The final pellets were suspended in a small volume of distilled water and lyophilized for further experiments.

3.8.6 Preparation of Spheroplast Cell Envelope

The cell envelopes was obtained according to procedure of section 3.8.5, and was resuspended in a 0.1 M pH 7.0 TRIS buffer with 20000U L^{-1} lysozyme. The suspension was incubated at 25°C with well shaking for destructing and removing cell PEG layer. After a 60min incubation, the cell pellets were collected by centrifugation at 18 000 g for 60 min, and the pellets were then washed twice with 0.05 M pH 7.2 HEPES buffer, twice with distilled water. The final pellets were considered as spheroplast cell envelope material and were suspended in a small volume of distilled water and lyophilized for further experiments.

3.8.7 Separation of Outer Membrane

The cell envelopes was obtained according to procedure of section 3.8.5, and then the envelopes were resuspended in a 0.05 M pH 7.0 TRIS buffer with 20000 U L^{-1} lysozyme. The suspension was incubated at 25°C with well shaking for destructing and removing cell PEG layer. After a 60min incubation, the cell pellets was collected by centrifugation at 18000 g for 60 min, and the pellets were then washed by 0.1M pH 6.5 HEPES buffer containing 0.5% TritonX-100, 20 mM Mg^{2+} for 30min. The pellet after centrifugation at
18000 g for 60 min was obtained and washed twice with 0.05 M pH 7.2 HEPES buffer, twice with distilled water. The final pellets were considered as outer membrane material and were suspended in a small volume of distilled water and lyophilized for further experiments.

3.8.8 Preparation of Peptidoglycan Layers from Cell Envelopes

Isolated envelopes were extracted with 1% sodium dodecyl sulfate (SDS) solution for 30 min at 25°C, then the suspensions were centrifuged for 60 min at 25000 g to separate the PEG layer materials. The pellets were then washed with 0.2% SDS, and three times with 1 mol L⁻¹ NaCl, five times with distilled water [Weidel and Peltzer, 1964; Beveridge et al. 1982]. The final pellets were considered as cell PEG layer material and were suspended in a small volume of distilled water and lyophilized for further experiments.

3.8.9 Determination of Content of Metal Binding Related Activity Ingredients on Cell Surface

1. Protein Content

Proteins content on cell surface was determined by the Folin method [Lowry, 1951], at 595 nm with bovine serum albumin as standard after extraction with 90% dichloromethane.

2. Phospholipid Content

Determination of total lipid phosphorus was through digestion of the sample to yield inorganic phosphate. Subsequent treatment of the digest with a reagent produces a colored product. Digestion reagent is 70% HClO₄ and a suitable color reagent is
aminonaphthoisulfonic acid (ANSA). A blue color develops as a result of the interaction of inorganic phosphate and the ANSA. [Hanahan, 1996].

Assay procedure:

Pre-purified phospholipid containing sample containing lipid P in the range of 1-5 μg was transferred into an acid-washed Pyrex digestion tube. 70 % reagent grade perchloric acid was added into the tube, and placed in a sand bath maintained 180°C for 2 hr. A set of potassium dihydrogen phosphate standards was subjected to the same treatment as the unknown lipid sample. The digested samples then were treated with the ANSA reagent, and the mixture is placed in a boiling water bath until the blue color has stabilized at a maximum-usually 20 min. The absorbance of the samples (including the standards) was read at 830 nm. Then a working graph is prepared using the absorbance of the standards plotted against the phosphorus concentration of the standards. The concentration of the phosphorus in unknown lipid samples could be obtained from the working graph by using their absorbance values. Finally, the phospholipid content in cell membrane could be calculated assuming an average molecular weight of 700 [Osborn, et al, 1972].

3. Lipopolysaccharide content in outer membrane

KDO (2-keto-3-deoxyoctonate) was determined for estimating Lipopolysaccharide content in outer membrane by the method Karkhanis,(1978).

Assay Procedure:

(i) Add 1 ml of 0.2 N H₂SO₄ to a test tube containing 1-2 mg of LPS- containing material.

(ii) Heat the reaction mixture at 100°C for 30 min, cool and centrifuge at 15000 g for 5 min, and pipet 0.5 ml as a clear solution into another test tube.
(iii) Add 0.25 ml of 0.04 M HIO dissolved in 0.125 N H₂SO₄, vortex, and allow the mixture stand at room temperature for 20 min.

(iv) Add 0.25 ml of 2.6 % NaAsO₂, in 0.5 M HCl, vortex, and wait until the brown colour disappears.

(v) Add 0.5 ml of 0.6 % thiobarbituric acid (TBA), vortex, and beat the mixture at 100°C for 15 min. While hot, add 1 ml of dimethylsulfoxide (DMSO). Let the mixture cool to room temperature and read its optical density at 548 nm against a blank, treated as above, without KDO.

The amount of KDO in the material is calculated from the standard curve, optical density versus concentration, using the following formula:

\[
\text{Amount of KDO per milligram of material} = \frac{2 \times \text{amount calculated from the graph}}{\text{amount of starting material}}
\]

Finally, the lipopolysaccharide (LPS) content in cell outer membrane could be calculated by estimating the KDO was average 4.7 % of the weight of LPS (Richard and Robert, 1983)

To obtain a standard curve, volumes of known concentrations of KDO from the stock solution (0.1 mg ml⁻¹) were taken in 0.5 ml of 0.2 N H₂SO₄ instead of 1 ml and treated as above. Following hydrolysis at 100°C for 30 min, the reaction mixture was treated with HIO, without centrifugation. The optical density at 548 nm was corrected for the total volume of 2.5 ml.

4. PEG Content

Diaminopimelic acid (DPA) was determined by Ninhydrin method of Work (1957) for estimating PEG content in PEG layer materials.
Assay Procedure

DPA containing material was digested with 2 ml 0.5M H₂SO₄ at 160°C for 1 hour. After digestion the sample volume was recovered to 2.0 ml by 0.5 ml H₂SO₄, then 0.5 ml sample was mixed with 0.5 ml HAc and 0.5 ml Ninhydrin reagent. The mixture was covered and heated in a bath at 100°C for 1 hour, then cooled to room temperature. 3.5 ml HAc was added to bring the final volume to 5 ml, and read its optical density at 440 nm against a blank. A reagent blank only containing water and a set of standard DPA sample was treated simultaneously. The PEG contents could be finally calculated by estimating the DPA was average 18.3% of the weight of PEG [Weidel and Pelzer, 1964].

Ninhydrin reagent: 1g Ninhydrin dissolved in 40ml 0.6M H₃PO₄
Chapter 4. Screening and Preparation of Bacterial Cell as Biosorbent for Binding Copper and Nickel

From practical operation point of view, biosorbent regeneration, transport and disposal contributed to major cost comparing with biosorbent production in biosorption process [Atkinson et al., 1998]. In order for biosorption to become competitive with existing technologies, metal adsorption capacity of biomass should be improved as far as possible to reduce the amount of biosorbent used, thus minimize the sludge regeneration, disposal and transport cost. For improving adsorption capacity of biosorbent for Cu$^{2+}$ and Ni$^{2+}$, the screening technology for selecting bacteria with high Cu$^{2+}$ and Ni$^{2+}$ adsorption capability should be developed. Furthermore, the optimization of biosorbent production techniques should be widely studied.

4.1 Screening of Bacteria with High Copper and Nickel Binding Capability

33 bacterial strains were isolated from heavy metal contaminated sites (for example: electroplating effluent) and 27 Cu$^{2+}$ resistant bacterial strains were obtained from activity sludge, respectively. Experiments showed that the Cu$^{2+}$ resistant bacteria and the bacteria isolated from electroplating effluent appeared obvious growth in LB medium containing 100 mg L$^{-1}$ Cu$^{2+}$, while Cu$^{2+}$ unreistant bacteria isolated from activity sludge hardly grew in the same medium.

The bacterial strains with high Cu$^{2+}$ binding capacities were pre-selected from these 60 bacterial strains using plate quickly screening method following the procedure of section 3.1.4. Total 41 strains, in which 26 strains came from electroplating effluent and 15
strains from Cu$^{2+}$ resistant bacteria, were found to have visual black haloes around the strain colony with diameter of above 1cm on SM plate containing 20 mg L$^{-1}$ Cu$^{2+}$ after spraying by 2 % (NH$_4$)$_2$S solution. The black haloes resulted from the formation of CuS precipitation (Figure 4.1) indicated that these strains had the capability of accumulating Cu$^{2+}$ to its surrounding zone. Thus the 41 bacterial strains were tentatively considered as high Cu$^{2+}$ binding capacity bacteria, and were picked out for further determination of the Cu$^{2+}$ binding capacity by method of section 3.4.1. Quantitative testing results showed that the Cu$^{2+}$ adsorption capacities of 26 pre-selected strains isolated from electroplating effluent were all above 33 mg g$^{-1}$. and therein 12 strains were found to be above 40 mg g$^{-1}$, (Figure 4.2a), while 17 Cu$^{2+}$ resistant bacteria were above 31.2 mg g$^{-1}$, and 7 strains were found to be above 40 mg g$^{-1}$, (Figure 4.2b). As control, the Cu$^{2+}$ adsorption capacities of heavy metal non-resistance bacteria isolated from activity sludge were found to be 25 mg g$^{-1}$ below.

The 19 strains with Cu$^{2+}$ binding capacity of more than 40 mg g$^{-1}$ were picked out and stored, from which the high Ni$^{2+}$ binding capacity bacteria were selected.

Pre-screening of Ni$^{2+}$ binding bacteria was carried from 19 high Cu$^{2+}$ binding bacteria mentioned above using quickly screening method on SM plate. 15 strains of these 19 Cu$^{2+}$ binding bacteria were found to have visual black haloes around its colony with diameter of above 1cm on Ni$^{2+}$ containing SM medium after spraying by 2% (NH$_4$)$_2$S solution. The Quantitative testing results showed that the Ni$^{2+}$ adsorption capacities of these 15 strains all were more than 23 mg g$^{-1}$, and 7 strain were found to be more than 30 mg g$^{-1}$, (Table 4.1). Therein 5 strains came from electroplating effluent and others came from Cu$^{2+}$ resistant bacteria. The 7 bacterial strains were considered to possess high Cu$^{2+}$ and Ni$^{2+}$ adsorption capacity simultaneously, and named as Strain E1, E2, E3, E4, E5, and R1, R2, respectively.
Despite there are 7 strains with high Cu\(^{2+}\) and Ni\(^{2+}\) removal capacities, some of them possessed certain undesirable characteristics. For example, strain E1 and E3 grew very slowly, while E2, E4, R1, R2 were difficult to be separated from culture medium or wastewater by settling due to producing excess slime. Among them the strain E5 with Cu\(^{2+}\) and Ni\(^{2+}\) binding capacities of 44.7 mg g\(^{-1}\) and 32.2 mg g\(^{-1}\), respectively, was selected for further studies.

Resistance to heavy metals was usually expected to correlate with the microorganisms extraordinary 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]. This is because heavy metal resistant bacteria have a series of functions to reduce the toxicity of heavy metal ions for maintaining cell growth and metabolism, such as metabolism-mediated oxidation-reduction, micro-precipitation and metal-linked proteins [Beveridge et al, 1997; White et al, 1995; Misra, 1992a; 1992b]. The functions of metal resistant bacteria for reducing the toxicity of heavy metal to maintain cell growth are also useful to sequester heavy metal from wastewater when the cell biomass is used as biosorbent.

In the present experiment, high heavy metal accumulating bacteria were obtained from heavy metal resistant bacteria or from bacteria isolated from heavy metal contaminated site (such as electroplating effluent).

The bacteria isolated from heavy metal contaminated site, in fact, might be considered as heavy metal resistant bacteria, because through long-term adaption in heavy metal contaminated environment, the bacteria have developed some adjusting functions to reduce toxicity of heavy metal as similar to heavy metal resistant bacteria. The results of cell growth in SM containing 100 mg L\(^{-1}\) Cu\(^{2+}\) mentioned above also confirmed that the bacteria isolated from electroplating effluent are Cu\(^{2+}\) resistant bacteria.
The pre-screening method used in the present study also select for heavy metal accumulating microorganisms quickly and directly by detecting the presence of Cu$^{2+}$ or Ni$^{2+}$ precipitate on the cell or its surrounding zone. (NH$_4$)$_2$S is a reagent that can react with Cu$^{2+}$ and Ni$^{2+}$ to form black precipitate [Thomas, 1995]. In the present study, it was found that some bacterial cells growing in semi-solid medium containing Cu$^{2+}$ or Ni$^{2+}$ produced obvious black haloes in its surrounding zone after spraying (NH$_4$)$_2$S. It was hypothesized that the diameter of black haloes was proportional to the Cu$^{2+}$ or Ni$^{2+}$ binding capacity of the bacteria. As shown in Figure 4.2, all of the bacterial colonies with black haloes of diameter above 1 cm in its surrounding zone were found to be Cu$^{2+}$ binding capacities of more than 30 mg g$^{-1}$. This showed that the screening method was really useful in the present study.

However, the correlation between diameter change of black haloes and Cu$^{2+}$ or Ni$^{2+}$ binding capacity, which would be meaningful if the screening program involves a very large number of bacteria, had not been statistically established in the present study. The concrete Cu$^{2+}$ or Ni$^{2+}$ binding capacities of bacterial cell isolated by the screening method were still determined by adsorption experiments.
Figure 4.1 Screening of bacteria with Cu\(^{2+}\) accumulation capability by Na\(_2\)S precipitation method
Figure 4.2a: Distribution of Copper Adsorption Capacity of Bacteria Isolated from Electroplating Effluent
Figure 4.2b: Distribution of Copper Adsorption Capacity of Copper Resistant Bacteria

Number of Bacteria

Copper Adsorption Capacity (mg/g)
**Table 4.1** Ni$^{2+}$ adsorption capacity and related features of different bacteria isolated from Cu$^{2+}$-binding bacteria

<table>
<thead>
<tr>
<th>Bacteria Strain</th>
<th>Ni$^{2+}$ Binding Capacity (mg g$^{-1}$)</th>
<th>Growth Rate</th>
<th>Settling Capability</th>
</tr>
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<tbody>
<tr>
<td>Strain E1</td>
<td>31.9</td>
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<td>--</td>
</tr>
<tr>
<td>Strain E2</td>
<td>32.3</td>
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<td>-</td>
</tr>
<tr>
<td>Strain E3</td>
<td>30.3</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>Strain E4</td>
<td>34.8</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Strain E5</td>
<td>32.2</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Strain R1</td>
<td>33.1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Strain R2</td>
<td>31.2</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
4.2 Characteristics of Strain E5

The characteristic of strain E5 isolated from electroplating effluent with high \( \text{Cr}^{2+} \) and \( \text{Ni}^{2+} \) binding capacity were identified by Biology Department of Chinese University of Hong Kong according to the classification scheme in Bergeys Manual [Holt, et al. 1994]. Strain E5 is a gram-negative bacterium, straight or slightly curved rods but not helical, 0.5-1.0 \( \mu \text{m} \) in diameter by 2.0-4.0 \( \mu \text{m} \) in length. It does not accumulate poly-\( \beta \)-hydroxybutyrate in cell. It does not produce prosthecæ and is not surrounded by sheaths. It is motile with polar flagella (Figure 4.3). Growth occurs from 4°C to 40°C. The colonies formed by this strain are smooth, circular, convex and ivory, and appear a diameter of 1-2.5 mm after culturing for 2 days on LB plate. However the morphology of colonies changes with the kind of medium or with culture age.

It is facultatively aerobic, having a respiratory type of metabolism with oxygen as the terminal electron acceptor. In some case, however, nitrate can be used as an alternate electron acceptor, allowing growth to occur anaerobically. Xanthomonadins are not produced. The strain can grow in high salt containing medium (7% NaCl), but fail to grow under acid conditions (pH 4.5). It is oxidase-positive, catalase-positive, and chemoorganotrophic.

The strain is able to utilize citrate, glucose and maltose as well as arginine, L-valine. However, it was unable to utilize geraniol, trehalose and inositol. Some characteristics of physiology and biochemistry of strain E5 are listed in (Table 4.2).

Base on these characteristics, strain E5 was tentatively identified as a gram-negative \textit{Pseudomonas Putida}, and was named as \textit{Pseudomonas Putida} 5-x.

In previous studies, gram-positive bacteria had been thought to possess higher heavy metal binding capacity than gram-negative bacteria due to their thicker PEG layer in
gram-positive cell wall than in gram-negative cell envelopes [Brierley 1990a; McLean and Beveridge, 1990]. However, in the present study, a gram-negative *Pseudomonas Putida* was found to be high Cu²⁺ and Ni²⁺ binding capacity, thus it attracted us to further study its heavy metal adsorption properties and adsorption mechanisms.
Figure 4.3 *Pseudomonas putida* 5-μ cell with polar flagellum (bar = 1.0μm)
Table 4.2 Characteristics of physiology and biochemistry of strain E5

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Strain E5</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of flagella</td>
<td>1-2</td>
</tr>
<tr>
<td>Fluorescent pigments</td>
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<td>Pyocyanin</td>
<td>--</td>
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<td>Carotenoids</td>
<td>--</td>
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<td>Growth at 41°C</td>
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<tr>
<td>Levan formation from sucrose</td>
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<tr>
<td>Arginine dihydrolase</td>
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<td>Oxidase reaction</td>
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</tr>
<tr>
<td>β-Alanine</td>
<td>+</td>
</tr>
<tr>
<td>DL-Arginine</td>
<td>+</td>
</tr>
<tr>
<td>Citrate</td>
<td>++</td>
</tr>
</tbody>
</table>
4.3 Optimization of Cell Preparation as Biosorbent for Copper and Nickle

4.3.1 Effect of Nutrient Limitation

Since most biosorption processes involve the microbial cell surfaces. When change the culture conditions of microbial cells like growth rate, medium composition, incubation temperature etc., the cell surface properties and components such as the contents of protein, PEG, phospholipid and lipopolysaccharide would be influenced [Hancock and Poxton, 1988]. Composition of culture medium used affected cell surface properties of bacteria and thus the metal removal capacity of the bacterial cells were documented [Kwok, 1990; Huang et al., 1989].

In this study, five culture media listed in Table 3.1 were used for culturing P. putida 5-x cell as biosorbent, and the adsorption efficiency of cell biomass cultured in different media were assessed. Figure 4.4 showed the effect of nutrient limitation on Cu\(^{2+}\) and Ni\(^{2+}\) binding capacity of P putida 5-x cell. For Cu\(^{2+}\) binding, the P. putida 5-x cell cultured in sulfate and ammonia limiting medium had evidently high adsorption capacity compared with in other media, but for Ni\(^{2+}\), no evident differences were found except for phosphate and glucose limiting medium. Due to the growth rate of P. putida 5-x cell in ammonia limiting medium was obviously slower than in sulfate limiting medium (Table 4.3), from the view of the economical and practical point, the sulfate limiting medium was thus used for cell production in further investigation.

As above discussions, composition of culture medium might affect cell surface properties of bacteria, so the metal adsorption capacity of the bacterial cells. In normal case, or rather, in nutrition non-limiting medium, there is a balance among C, N, P and S in bacterial cell, the ratio of C: N: P: S in bacterial cell composition is around 50:14:3:1,
Howard et al., 1985]. However, in nutrition limiting medium, normal cell metabolism would be affected, hence the cell composition and structure would change. For example, in ammonia limiting or sulfate limiting medium, due to lack of N and S source, normal cell synthesis and metabolism would be limited, thus the over-C source or P source absorbed by cell would be converted forcibly to some carbon compounds and phosphour compounds in cell, such as polysaccharide and poly-phosphate, which are cell growth, metabolism unnecessary [Hong et al., 2000]. The carboxyl groups attached on polysaccharide and phosphonate groups attached on polyphosphate or phospholipid were major heavy metal binding site on bacterial cell due to their obviously negative charge [Schiewer and Volesky, 2000]. Thus, in the cells cultured in N or S limiting media, the polysaccharide and poly-phosphate was over-accumulated due to the lacking of N and S in medium, thus the cells took advantage of heavy metal binding.

However, in present study, the effect of nutrition-limiting media was just preliminary investigated, and the S-limiting medium might still not provide optimal ratio of C: N: P: S for P. putida 5-x cell production as biosorbtent. The experiment just made an exploration for finding relationship between medium composition and negative groups content heavy metal binding related. We hope further study on optimising the ratio of C: N: P: S in medium and using gene recombination technique to increase negative-charge group content on cell surface, and on synthesis mechanism of those heavy metal binding related negative-charge groups will be widely conducted in the future.

In previous studies, 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]. Kwok (1990) also found that nutrition limitation could affect the nickel removal capacity of an Enterobacter sp., but the effect of sulfate limitation on adsorption capacity of cell biomass was not investigated.
Figure 4.4 Effect of culture media on copper and nickel binding

- Copper Binding (mg/g)
- Nickel Binding (mg/g)
Table 4.3 Effect of Media Composition on Cell Growth

<table>
<thead>
<tr>
<th>Media</th>
<th>Cell Growth (O.D(_{600}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient Non-Limiting</td>
<td>5.80</td>
</tr>
<tr>
<td>Ammonia Limiting</td>
<td>2.09</td>
</tr>
<tr>
<td>Sulfate Limiting</td>
<td>5.65</td>
</tr>
<tr>
<td>Phosphate Limiting</td>
<td>4.95</td>
</tr>
<tr>
<td>Glucose Limiting</td>
<td>2.55</td>
</tr>
</tbody>
</table>

* Cells were cultured in different media as procedure of section 3.3.3, after culturing 36 h, the O.D\(_{600}\) of culture was tested for cell growth
4.3.2 Effect of Cell Growth Age

The effect of cell growth age on heavy metal adsorption capacity of cell biomass, and the optimal cell harvest phase during the *P. putida* 5-x cell production as biosorbent was studied. From Figure 4.5, it was found that *P. putide* 5-x cell growth in SLM could be classified to 4 major phases as follows: lag growth phase (0~6 h), logarithmic growth phase (10~22 h), stationary growth phase (24~34 h), and death phase (38~42 h). The amount of cell biomass was found to be maximum at 32~34 h. The Cu$^{2+}$ (or Ni$^{2+}$) adsorption capacity of *P. putida* 5-x cell in different growth age was also determined in solution containing 50 mg L$^{-1}$ Cu$^{2+}$ (or 46 mg L$^{-1}$ Ni$^{2+}$). It was clear that the adsorption capacities of cell biomass to Cu$^{2+}$ and Ni$^{2+}$ were different along with cell growth age. The variation tendencies of Cu$^{2+}$ and Ni$^{2+}$ binding capacity along with cell culture age were slightly different. When cell growth entered to logarithmic-growth phase, both Cu$^{2+}$ and Ni$^{2+}$ adsorption capacities of cell biomass fell off sharply. For Cu$^{2+}$, however, the lowest point of 24.3 mg g$^{-1}$ appeared at around 22-24 h, which is the end of logarithmic-growth phase. When cell growth entered into stationary growth phase, Cu$^{2+}$ adsorption capacity of cell began to increase, and accelerated in the later stationary-growth phase. Between the end of stationary growth phase and early cell death phase, (34~38 h), the adsorption capacity of *P. putida* 5-x cell to Cu$^{2+}$ reached maximum of 67.3 mg g$^{-1}$, and then fell off slowly again in the death phase, (Figure 4.5). For Ni$^{2+}$, the lowest point of 17.6 mg g$^{-1}$ appeared at around 16-18 h, which is mid-logarithmic-growth phase, when cell growth entered to later logarithmic-growth phase, the Ni$^{2+}$ adsorption capacity began to increase firstly, and the highest point of 33.8 mg g$^{-1}$ appeared at 28-30 h, which was mid-stationary growth phase.

Heavy metal ions bound on cell surface can form high electron-dense zone. Therefore TEM analysis enables the comparison of Cu$^{2+}$ bound cell surface appearance in different growth age.
(18 h, 32 h and 40 h). After Cu²⁺ uptake in some conditions, early cell surface showed image which was slightly discerned because of the lack of contrast of high electron-dense with the cell body, late cell of 32 h and 40 h exhibited an high electron-dense staining on cell surface due to large Cu²⁺ binding, (Figure 4.6). This result implied that Cu²⁺ was absorbed on cell surface, and the heavy metal binding sites (e.g. some negative charged carboxylate and phosphonate groups) on surface of late age cell were much more than that of early cell. The similar TEM analysis results also could be found on Ni²⁺ binding by P. putida 5-x cell with different growth age.

X-ray energy dispersion analysis (EDAX) showed that there were basic structure components like phosphorus and sulfur as well as trace Cu²⁺ on the surface of P. putida 5-x cell before Cu²⁺ adsorption (Figure 4.7). However, the amounts of P and S ligands on cells surface harvested in different periods were different. Figure 4.7 showed there were the most amounts of P ligands on cell surface harvested in 34h. The spectra displayed in Figure 4.8 showed clearly that the amount of Cu²⁺ on the biomass surface increased after exposure to Cu²⁺ containing solution. The conspicuous Cu²⁺ peak on the EDAX spectra in Figure 4.8 indicates that Cu²⁺ might deposit on the bacterial cell surface. According to the peak height of Cu²⁺ in EDAX spectra, it could be found that cell harvested in 34 h adsorbed the most amount of Cu²⁺, while cell harvested in 16h adsorbed the least amount of Cu²⁺. The EDAX result was consistent with the results in Figure 4.5 and Figure 4.6 that the adsorption capacity of cell was various along with cell age. The results of Figure 4.7 and Figure 4.8 implied that the amount of Cu²⁺ adsorbed by cell surface related to the amount of P and S ligands on cell surface. The more the P ligands on cell surface, the more the Cu²⁺ adsorbed on cell surface.
Figure 4.5 Copper and Nickel Binding Capacity by Cell Biomass in Different Growth Age

- **Ni²⁺ Uptake (mg/g)**
- **Copper Uptake (mg/g)**
- **Cell Biomass (OD600)**

![Graph showing binding capacity over cell culture times](attachment:binding_capacity_graph.png)
Figure 4.6: TEM graphs of *P. putida* 5-x cell surface appearance in different growth age after uptake of Cu$^{2+}$.
A: 16h Cell

B: 34h Cell

C: 40h Cell

Figure 4.7 X-ray analysis results of fresh *P. putida* 5-x cell in different cell growth phases without contacting Cu²⁺.
Figure 4.8 X-ray analysis results of fresh *P. putida* 5-x cell in different cell growth phases after contacting Cu$^{2+}$. 
Previously, the change in metal biosorption capacities with the culture age was also observed in some experimental studies. [Gilbert et al. 1991; Chang et al. 1997]. It had been reported by James (1982), Kuyucak and Volesky (1988), Lee and Bradley (1998), Pavel et al. (1999), that electrostatic interactions between cationic metal species and the negatively charged active groups on cell surfaces, such as carboxyl, phosphonate, amine and hydroxyl attached on peptidoglycans, lipopolysaccharides, phospholipids and proteins, might be responsible for biosorption of metals. The nature and composition of surface of some bacterial species can vary with growth age and the composition of growth medium [James, 1982], thus the amount of negative charge groups on cell surface and heavy metal binding capacity were various in different growth age. The present experimental results seemed to indicate that the content of some major active matter heavy metal binding related was various along with growth age. Because the variation tendencies of Cu$^{2+}$ and Ni$^{2+}$ binding capacity along with cell culture age were slightly different, Ni$^{2+}$ binding related active matter seemed to be slightly different from Cu$^{2+}$ binding related.

Comprehensive considerations of amount of cell biomass and Cu$^{2+}$ or Ni$^{2+}$ adsorption capacity, or rather, in term of total Cu$^{2+}$ or Ni$^{2+}$ adsorbed by cell biomass in one liter culture medium, the optimal harvest period in P. putida 5-x cell production as biosorbent for Cu$^{2+}$ and Ni$^{2+}$ was at around 34-36 h and 28-32 h, respectively.

4.3.3 Effect of Incubation Temperature

The effects of incubation temperature on Cu$^{2+}$ and Ni$^{2+}$ binding capacity were studied. P. putida 5-x cells were cultured at 30°C, 32°C and 37°C, respectively, the cells in different growth age were harvested, and the Cu$^{2+}$ and Ni$^{2+}$ binding capacities of cell biomass were tested. The results in Figure 4.9a showed that generally, bacterial cells
incubated at 37°C had the lowest Cu²⁺ and Ni²⁺ binding capacity in all growth age, particular in logarithmic growth phase compared with incubated at 30 and 32°C. For Cu²⁺ binding, the lowest point of adsorption capacity of cell biomass cultured at 37°C was 18.4 mg g⁻¹ at around 22h, but in 32°C or 30°C, was 24.3 mg g⁻¹ and 24.7 mg g⁻¹ at 22-24 h, respectively. However, the highest points of Cu²⁺ binding capacity by cell biomass incubated at 37°C, 32°C and 30°C had not obvious difference, that was 63.2 mg g⁻¹ at 34 h, 67.3mg g⁻¹ at 34-36 h, and 67.1 mg g⁻¹ at 34-36 h, respectively. Similar results were obtained on Ni²⁺ adsorption process as in Figure 4.9b. These results indicated that higher incubation temperature might inhibit the formation of some active matter heavy metal binding related, especially to the cell in logarithmic growth phase. Therefore, in further study, the cell culture temperature will be determined at 30-32°C.

It had been all known that higher incubation temperature could enhance cell growth rate and cell respiration, so that declined the accumulation of some matter in cell, such as polysaccharides, phospholipids and proteins, which were thought to be heavy metal binding related, on cell surface. In the end of stationary growth phase, the effects of temperature on cell growth rate and respiration decreased, and the active matter heavy metal binding related accumulated largely.

Gill (1975) and Bhakoo and Herbert (1980) reported that incubation temperature affected the phospholipid content and composition in bacterial cell of Pseudomonas spp. In another case, Andrew et al (1987) found growth temperature affected the proteins and lipopolysaccharides content in cell envelopes of Pseudomonas aeruginosa. Our experimental results seemed to be compatible with the admitted principle above mentioned.

Above experiments showed that suitable culture conditions could enhance Cu²⁺ (or Ni²⁺) binding capacity of P. putida 5-x., but the investigated conditions are by no means
exhaustive and a much more manipulations are possible. In the following discussions, the term "optimal conditions" is referred to culturing the *P. putida* 5-x in the sulfate limiting medium at 32°C for 34-36 hours for Cu²⁺ biosorbent, and 28-30 h for Ni²⁺ biosorbent.

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.
Figure 4.9a Effect of Incubation Temperature on Copper Binding Capacity of P. putida 5-x Cell

![Graph showing the effect of incubation temperature on copper binding capacity. The graph has a y-axis labeled 'Binding Capacity (mg/g)' and an x-axis labeled 'Cell Culture Time (hour).']
Figure 4.9b Effect of Incubation Temperature on Nickle Binding Capacity of *P. putida* 5-x Cell

![Graph showing the effect of incubation temperature on the nickel binding capacity of *P. putida* 5-x cell.](image)

- **Y-axis:** Binding Capacity (mg/g)
- **X-axis:** Cell Culture Times (hour)
- **Lines:**
  - 30C
  - 32C
  - 37C
4.3.4 Effect of Cell Pretreatment on Copper and Nickle Binding Capacity

Pretreatment with different chemical eluents or heating may change the cell surface structure, thus affect the heavy metal binding capacity.

Different chemical eluents and heating at 120°C were used to pretreat *P. putida* 5-x cell. The comparisons for Cu\(^{2+}\) and Ni\(^{2+}\) adsorption capacities of fresh *P. putida* 5-x cells with those pretreated cells showed that pretreated cells with diluted acid or (NH\(_4\))\(_2\)SO\(_4\) could enhance the adsorption capacity by 11-26 % for Cu\(^{2+}\) and 10-31 % for Ni\(^{2+}\). However, alkali and concentrated acid decreased both Cu\(^{2+}\) and Ni\(^{2+}\) adsorption capacity, it might be due to complete destruction of cell surface components and structure. (Table 4.4 and 4.5). The pretreatment by heating at 120°C seemed to not affect the heavy metal binding capacity of *P. putida* 5-x cell biomass.

A good pretreating solution must not result in a high biomass loss rate (BLR) in the cell pretreatment procedure. The effects of pretreatment by different eluents on BLR were also studied. The experimental results in Table 4.4 and Table 4.5 showed that the BLRs were <10 % when the diluted HCl (0.3 mol L\(^{-1}\) below) or (NH\(_4\))\(_2\)SO\(_4\) solutions were used as the eluent. NaOH, H\(_2\)SO\(_4\) solutions and 0.6 mol L\(^{-1}\) HCl were not better eluants for pretreating cell biomass since significant weight loss. Therefore, diluted acid such as 0.1~0.3 mol HCl was chosen in following experiments in order to avoid significant modification of the cell wall structure and to prevent considerable biomass weight loss. Previous studies also reported the effect of pretreatment on metals adsorption capacity of cell biomass [Kuyucak and Volesky, 1989c; Huang and Huang, 1996; Fourest and Roux, 1992].

9.11 g of pretreated cell were obtained from 10g fresh cells by treating with 0.1 mol L\(^{-1}\) HCl. Experimental results indicated that pretreatment by 0.1 mol L\(^{-1}\) HCl increased the
Cu^{2+} adsorption capacity of *P. putida* 5-x cells from 67.4 mg g\(^{-1}\) to 84.3 mg g\(^{-1}\), and the total Cu\(^{2+}\) bound has increased from 674 mg by 10 g fresh cells to 768 mg by 9.11 g HCl pretreated cells.

A loose superficial layer outside of the *P. putida* 5-x fresh cell was examined by TEM analysis, but after treating with 0.1 mol L\(^{-1}\) HCl, the degradation of the loose superficial layer was observed, (Figure 4.10). This indicated that enhanced adsorption of Cu\(^{2+}\) and Ni\(^{2+}\) by pretreated cells related to the degradation of the loose superficial layer outside of the fresh cell.

Bacterial capsule is a superficial layer of Gram-negative cells. It is highly hydrated (>95%), loosely arranged homopolymers or heteropolymers of carbohydrate and protein. These polymers are very flexible and extend up to several hundreds nanometer from the cell surface, entirely bathed in water and can be easily removed by weaker eluent [Geesey and Jang, 1989]. Theoretically, capsular carbohydrate and protein providing some electronegative groups such as carboxyl and hydroxyl groups may contribute to heavy metal ions binding, however, the contribution is limited due to its low electronegative groups density (>95% is water in capsule). In general, two electronegative groups are required to bind a divalent metal ion through metal salt-bridging [Geesey and Jang, 1989].

The presence of the metal salt-bridging result in a conformation change within the capsule, due to its highly hydrated and flexibility, might result in other metal-binding sites on cell outer membrane (much higher density than in capsule) become inaccessible to heavy metal binding, (Seeing Figure 4.11). Therefore, cells pretreated with 0.1 mol L\(^{-1}\) HCl could improve both Cu\(^{2+}\) and Ni\(^{2+}\) adsorption capacity due to degrading superficial layer (capsule) outside of the fresh cell.

In addition, acid pretreatment could be considered as cell regeneration process, the results in Table 4.6 showed that the native metal ions absorbed on cell surface during the culturing
in medium were desorbed by treatment of 0.1 mol L\(^{-1}\) HCl. Obviously, the removal of the impurities or native metal ions absorbed on binding sites in the \(P.\ putida\ 5-x\) cell biomass could also enhance the adsorption capacities to \(Cu^{2+}\) and \(Ni^{2+}\).
Table 4.4 Effect of pretreatment on Cu\(^{2+}\) adsorption capacity and biomass loss

<table>
<thead>
<tr>
<th>Eluents</th>
<th>Adsorption Capacity (mg g(^{-1}))</th>
<th>Increase Rate (%)</th>
<th>BLR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh Cell</td>
<td>67.3</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>0.1 mol L(^{-1}) HCl</td>
<td>83.6</td>
<td>24.2</td>
<td>9.7</td>
</tr>
<tr>
<td>0.3 mol L(^{-1}) HCl</td>
<td>81.8</td>
<td>21.5</td>
<td>9.9</td>
</tr>
<tr>
<td>0.6 mol L(^{-1}) HCl</td>
<td>57.1</td>
<td>-15.1</td>
<td>13.2</td>
</tr>
<tr>
<td>0.1 mol L(^{-1}) H(_2)SO(_4)</td>
<td>82.0</td>
<td>21.8</td>
<td>14.1</td>
</tr>
<tr>
<td>0.3 mol L(^{-1}) H(_2)SO(_4)</td>
<td>49.6</td>
<td>-26.3</td>
<td>16.9</td>
</tr>
<tr>
<td>0.6 mol L(^{-1}) H(_2)SO(_4)</td>
<td>43.8</td>
<td>-34.9</td>
<td>27.8</td>
</tr>
<tr>
<td>10 % (NH(_4))(_2)SO(_4)</td>
<td>74.9</td>
<td>11.3</td>
<td>8.8</td>
</tr>
<tr>
<td>15 % (NH(_4))(_2)SO(_4)</td>
<td>76.2</td>
<td>13.2</td>
<td>9.7</td>
</tr>
<tr>
<td>0.1 mol NaOH</td>
<td>51.6</td>
<td>-23.3</td>
<td>21.4</td>
</tr>
<tr>
<td>0.3 mol NaOH</td>
<td>42.8</td>
<td>-36.4</td>
<td>37.8</td>
</tr>
<tr>
<td>Heating at 120°C</td>
<td>62.1</td>
<td>-7.7</td>
<td>8.3</td>
</tr>
</tbody>
</table>

*Cu\(^{2+}\) adsorption capacities were determined in a pH 6.5 solution containing 50 mg L\(^{-1}\) Cu\(^{2+}\) and 0.5 g L\(^{-1}\) biosorbent. All data was average value of three experimental results.
<table>
<thead>
<tr>
<th>Eluents</th>
<th>Adsorption Capacity (mg g⁻¹)</th>
<th>Increase Rate (%)</th>
<th>BLR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh Cell</td>
<td>28.1</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>0.1 mol L⁻¹ HCl</td>
<td>36.8</td>
<td>31</td>
<td>9.7</td>
</tr>
<tr>
<td>0.3 mol L⁻¹ HCl</td>
<td>33.9</td>
<td>20.6</td>
<td>9.9</td>
</tr>
<tr>
<td>0.6 mol L⁻¹ HCl</td>
<td>24.1</td>
<td>-14.2</td>
<td>13.2</td>
</tr>
<tr>
<td>0.1 mol L⁻¹ H₂SO₄</td>
<td>33.7</td>
<td>19.9</td>
<td>14.1</td>
</tr>
<tr>
<td>0.3 mol L⁻¹ H₂SO₄</td>
<td>23.9</td>
<td>-14.9</td>
<td>16.9</td>
</tr>
<tr>
<td>0.6 mol L⁻¹ H₂SO₄</td>
<td>25.2</td>
<td>-10.3</td>
<td>27.8</td>
</tr>
<tr>
<td>10 % (NH₄)₂SO₄</td>
<td>31.2</td>
<td>11.0</td>
<td>8.8</td>
</tr>
<tr>
<td>15 % (NH₄)₂SO₄</td>
<td>30.8</td>
<td>9.6</td>
<td>9.7</td>
</tr>
<tr>
<td>0.1 mol NaOH</td>
<td>26.9</td>
<td>-4.3</td>
<td>21.4</td>
</tr>
<tr>
<td>0.3 mol NaOH</td>
<td>24.8</td>
<td>-11.8</td>
<td>37.8</td>
</tr>
<tr>
<td>Heating at 120</td>
<td>27.7</td>
<td>1.4</td>
<td>8.3</td>
</tr>
</tbody>
</table>

* Ni²⁺ adsorption capacities were determined in a pH 6.5 solution containing 46 mg L⁻¹ Ni²⁺ and 0.5 g L⁻¹ biosorbent. All data was average value of three experimental results.
Figure 4.10 TEM pictures of the cell surface of *P. putida* 5-x (x 30,000)
Figure 4.11. Simulative divalent Cu$^{2+}$ binding process by fresh cell and pretreated cell.
<table>
<thead>
<tr>
<th>Metals</th>
<th>Native Metal Bound on Cell Surface (mg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
<td>20</td>
</tr>
<tr>
<td>K</td>
<td>21.8</td>
</tr>
<tr>
<td>Mg</td>
<td>9.6</td>
</tr>
<tr>
<td>Ca</td>
<td>11.7</td>
</tr>
<tr>
<td>Mn</td>
<td>7.9</td>
</tr>
<tr>
<td>Ni</td>
<td>5.2</td>
</tr>
<tr>
<td>Cu</td>
<td>6.7</td>
</tr>
</tbody>
</table>

* All native metal was desorbed by 0.1 M HCl, and the native metal bound on cell was calculated by their concentration in desorbed solution.
Chapter 5: Study on Adsorption Characteristics and Adsorption Conditions

Although a good biosorbent may be used under a wide range of operating conditions, biosorption involves certain physico-chemical interactions between the metals and the cellular surface components and therefore is influenced by environmental condition that generally affects physico-chemical processes. Studies on the adsorption characteristics of biosorbent and the optimal adsorption conditions can serve as a useful aid for designing a suitable operation program to efficient remove heavy metal in practical application.

5.1 Copper and Nickle Uptake Kinetics

The Cu$^{2+}$ and Ni$^{2+}$ uptake kinetics of fresh and pre-treated *P. putida* 5-x were studied as procedure of section 3.4.2. Figure 5.1 depicts the time-course profiles for Cu$^{2+}$ and Ni$^{2+}$ biosorption with the initial Cu$^{2+}$ concentration at 50 mg L$^{-1}$ and Ni$^{2+}$ concentration at 46 mg L$^{-1}$ by 0.5 g L$^{-1}$ fresh suspended cells or pretreated cell of *P. putida* 5-x at solution of pH 6.5.

The experimental results in Figure 5.1 showed that for fresh cell, Cu$^{2+}$ and Ni$^{2+}$ removal started with a very rapid uptake process within the first 10~15 min followed by a stage of slow uptake lasting for about 120 min. About 80% of the total Cu$^{2+}$ and Ni$^{2+}$ taken up by the bacterial cells were removed within the first 10~15 min. For 0.1 mol L$^{-1}$ HCl pretreated cell, Cu$^{2+}$ and Ni$^{2+}$ adsorptions were finished rapidly within the first 10-20 min without following slower stage such as that of fresh cells.

According to the experimental results, we speculated that adsorption of Cu$^{2+}$ and Ni$^{2+}$ by fresh cell consists of two phases: a rapid adsorption phase followed by a slower
accumulating phase. The phenomenon was similar to the results reported by Khummongkol et al., (1982); Karamushkat et al. (1995) and Garnham et al. (1991). However, for pretreated cell, rapid phase, instead of slower uptake phases, followed a stationary phase, it could be due to the balance of diffusion and adsorption of metal ions on the bacterial cell surface [Huang et al. 1990]. This illustrated that the uptake kinetic of acid pretreated cell differed with fresh cell in Cu$^{2+}$ and Ni$^{2+}$ binding process.

The rapid kinetics of bi-phasic adsorption has significant practical importance, as it will facilitate smaller reactor volumes ensuring efficiency and economy. The adsorption process of pretreated cell to Cu$^{2+}$ and Ni$^{2+}$ was a typical mono-phasic adsorption of metals involving no energy-mediated reactions, where metal removal from solution is due to purely physio-chemical interactions between the biomass and metal in solution [Lo et al., 1999; Shuttleworth and Unz, 1993]. The uptake kinetics study indicated that pretreated cell of P putida 5-x could remove Cu$^{2+}$ and Ni$^{2+}$ more quickly than fresh cell if applied to treatment of industrial effluents, and the 15-20 min is enough to efficiently remove Cu$^{2+}$ and Ni$^{2+}$ from wastewater by adsorption process.

In broad terms, the biosorption process includes metabolism-independent passive biosorption process by living/dead cell, and metabolism-dependent bioaccumulation process just by dead cell. In practical application, metabolism-independent biosorption could simplify the operation procedure of adsorption process, for example, the toxicity of heavy metal in wastewater to cell biomass, the cell growth conditions during the biosorption process could not be considered. Thus the metabolism-dependency of rapid adsorption phase and slower phase (or stationary phase) should be confirmed for determining optimal adsorption phase and adsorption condition for efficient removal of Cu$^{2+}$ and Ni$^{2+}$ from electroplating effluent.
Figure 5.1. Copper and Nickle Uptake Kinetics

Copper-Pre -------- Cu$^{2+}$ adsorption by pretreatment cell

Nickle-Pre -------- Ni$^{2+}$ adsorption by pretreatment cell

Copper-Fre -------- Cu$^{2+}$ adsorption by fresh cell

Nickle-Fre -------- Ni$^{2+}$ adsorption by fresh cell
5.2 Effect of Glucose and Sodium Azide on Copper Removal Capacity

For determination of the metabolism-dependency of rapid uptake phase and followed slower uptake phase (or stationary phase), metabolism-accelerant glucose and metabolism-inhibitor sodium azide were used to assess the metabolism-dependency of bi-phase biosorption process.

2 % glucose and 0.2 % sodium azide was added into 50 mg L$^{-1}$ Cu$^{2+}$ containing buffer solution, and the adsorption processes by fresh cell and pretreated cell were observed and compared with control process without glucose and sodium azide, respectively. The results in Figure 5.2a showed that the presence of glucose during Cu$^{2+}$ adsorption seemed not to affect the Cu$^{2+}$ adsorption capacity of both fresh cell and pretreated cell in the rapid uptake phase, but slightly improved the Cu$^{2+}$ binding of fresh cell in slower uptake phase, particularly after 120 mins. The presence of sodium azide during the adsorption process obviously inhibited the Cu$^{2+}$ adsorption capacity of fresh cell in slower uptake phase, but no effect to rapid adsorption phase of both fresh cell and pretreated cell, and stationary phase of pretreated cell, (Figure 5.2b).

Glucose was used as an carbon source and energy source for bacterial metabolism while sodium azide was used as a metabolism inhibitor due to its inhibiting respiration [Townsley et al., 1986]. The results showed that in rapid uptake phase, fresh cells might not lose or enhance its Cu$^{2+}$ binding capacity even inhibiting its cell metabolism by sodium azide or improving its cell metabolism by glucose. This implied that Cu$^{2+}$ uptake of fresh cell in rapid uptake phase was probably metabolism-independent, or rather, it was a rapid physical-chemical adsorption process defined as “biosorption” in this thesis. However, experimental results showed that metabolism-accelerant glucose improved the Cu$^{2+}$ binding capacity of fresh cell in slower phase, while metabolism-inhibitor sodium
azide obviously decreased the Cu$^{2+}$ uptake in slower uptake phase. It had be well known that some cell metabolism actions were useful to heavy metal uptake, for example, some heavy metals are essential to microbial metabolism and they could be transported into the cytoplasm via specific ion pumps and transport system; cell oxidation and reduction system could oxidize or reduce inorganic compounds and accumulated them as insoluble compounds on cell surface; and cell metabolism could produce some compounds such as hydrogen sulfide which react with heavy metal ions to form insoluble metal sulfides, [Brierley et al., 1989; Nealsen et al., 1989; Hutchins et al., 1986; Ishibashi et al., 1990; Komori et al., 1990b]. These actions all could reduce the concentration of heavy metal ions in wastewater. Metabolism accelerant or inhibitor can improves or declines these metabolism actions heavy metal uptake related, hence affects the metabolism-dependent heavy metal uptake of cell. Above experimental results indicated that the slower uptake phase of fresh cell was metabolism-dependent uptake and defined as bioaccumulation in this thesis.

For 0.1 mol L$^{-1}$ HCl pretreated cells, glucose and sodium azide did not affect its Cu$^{2+}$ uptake capacity both in rapid phase and stationary phase, this implied that through acid pretreatment, the cell was killed, and cell metabolism stopped, the Cu$^{2+}$ uptake in all phases of pretreated cell adsorption process was metabolism-independent.

In practical application, metabolism-independent biosorption process could simplify the operation procedure and speed adsorption process. Both fresh cell in metabolism-independent rapid uptake phase and metabolism-independent pretreated cell could be used in removal of heavy metal from wastewater by adsorption process. However, for fresh cell, the Cu$^{2+}$ adsorption capacity was relatively low compared with pretreated cell, and only 80% of heavy metal ions were removed by metabolism-independent rapid uptake process within the first 10~15 min, thus, 0.1 mol L$^{-1}$ HCl pretreated cell seemed to
be suitable as biosorbent for heavy metal removal.

Figure 5.2a Effect of Glucose on Copper uptake
Kinetic of Fresh Cell and Pretreated Cell

Copper-Pre  Cu$^{2+}$ adsorption by pretreatment cell
Copper-Pre+G Cu$^{2+}$ adsorption by pretreatment cell with glucose
Copper-Fre Cu$^{2+}$ adsorption by fresh cell
Copper-Fre+G Cu$^{2+}$ adsorption by fresh cell with glucose
Figure 5.2b. Effect of Sodium Azide on Copper uptake
Kinetic of Fresh Cell and Pretreated Cell

Copper-Pre  Cu^{2+} adsorption by pretreatment cell

Copper-Pre+G  Cu^{2+} adsorption by pretreatment cell with azide

Copper-Fre  Cu^{2+} adsorption by fresh cell

Copper-Fre +G  Cu^{2+} adsorption by fresh cell with azide
5.3 Effect of pH On Copper and Nickle Adsorption Capacity

Since biosorption can dramatically change with pH, this key parameter should always be reported. Data are comparable only if they are obtained at the same pH. In this study, the effect of solution pH on Cu\(^{2+}\) and Ni\(^{2+}\) adsorption capacity was studied. As shown in Figure 5.3, there was a general increase in Cu\(^{2+}\) and Ni\(^{2+}\) adsorption capacity in accordance with an increase in pH, but after pH increasing to 10 and 11, the adsorption capacity to Cu\(^{2+}\) and Ni\(^{2+}\) decreased again, respectively. For Cu\(^{2+}\), the increase was very pronounced at pH 6-9, but for Ni\(^{2+}\) the increase was gentle along with pH increasing from 4.5 to 10. When at pH 9 solution, the adsorption capacity was up to almost three-folds of that at pH 5.5. Since biosorption predominantly is a physio-chemical process taking place between positively charged metal ions and anionic groups of cell surface [Muraleedharan et al., 1991], metal removal would be strongly influenced by the experimental conditions such as solution pH, specific surface properties of adsorbent, concentration of adsorbate and the presence of cations in solutions. It has been consistently reported that pH is the dominant solution parameter controlling biosorption. The solution pH not only affects the solution chemistry of the metals, but also governs the activity of functional groups in the biomass as well as the competition of metallic ions for the binding sites [Kuyucak and Volesky, 1988].

It is very likely that hydrogen ions compete with metal ions for the sorption sites of cells [Zhang et al., 1998]. At low pH the competition of hydrogen ions with Cu\(^{2+}\) and Ni\(^{2+}\) for binding sites might have caused the decrease of Cu\(^{2+}\) and Ni\(^{2+}\) removal capacity of \textit{P.putida} S-x. but at higher pH, more Cu\(^{2+}\) or Ni\(^{2+}\) ions would be taken up due to hydrogen ions decreased.

In addition, at low pH solution, some active groups metal binding related, such as hydroxyls, amides and amines attached on proteins and polysaccharides, are
electropositive, but in higher pH those groups become electronegative for efficiently binding positively charged heavy metal [Schiewer and Volesky, 2000].

At above pH 6.5, the Cu$^{2+}$ adsorption capacity increased sharply, this might be due to the formation of insoluble copper hydroxide precipitate largely deposited on cell surface. It was known that at pH around 6 of solution, Cu$^{2+}$, CuOH$^+$, Cu$_2$(OH)$_2$$^{2+}$, Cu(OH)$_2$ and Cu(OH)$_4$$^{2-}$ may be present. However, Cu$^{2+}$ was a predominant ion [Stumm and Morgan, 1995]. The concentration ratios of CuOH$^+$, Cu(OH)$_2$ and Cu(OH)$_4$$^{2-}$ to Cu$^{2+}$ at around pH 6 are 0.01, 5.01*10$^{-9}$ and 3.98*10$^{-16}$, respectively [Stumm and Morgan, 1995]. But at higher pH solution, the CuOH$^+$, Cu$_2$(OH)$_2$$^{2+}$, Cu(OH)$_3$ became predominant and result in the formation of insoluble copper hydroxide precipitate.

According to above experimental results, for heavy metal removal from wastewater, higher pH was advantage over low pH. However, the Cu$^{2+}$ and Ni$^{2+}$ adsorbed by cell biomass at higher pH solution were more difficult to be desorbed and recovered, thus the regeneration and reuse of cell biomass was not efficient, thus adsorption process carried at over high pH should be avoided in practical application.
Figure 5.3: Effect of pH on Copper and Nickel Adsorption capacity of Pretreated Cell of P. putida 5-x
5.4 Effect of Metal Ions on Copper and Nickel Adsorption Capacity

In electroplating effluent of Hong Kong, Ni$^{2+}$, Cu$^{2+}$, Zn$^{2+}$ and Pb$^{2+}$ are commonly found, thus in this study, the effect of other three cations with equi-molar concentrations on Cu$^{2+}$ (or Ni$^{2+}$) adsorption capacity were investigated. The results in Figure 5.4a presented that the Ni$^{2+}$ and Zn$^{2+}$ seemed not to affect Cu$^{2+}$ uptake, while Pb$^{2+}$ reduced the Cu$^{2+}$ removal capacity by about 3 folds. However, The presence of Cu$^{2+}$, Zn$^{2+}$ and Pb$^{2+}$ all obviously inhibited the Ni$^{2+}$ adsorption, respectively (Figure 5.4b). In addition, when all four metals co-existed, the inhibition influences on Cu$^{2+}$ and Ni$^{2+}$ biosorption by *P. putida* 5-x did not accumulate proportionally.

Experimental data in Figure 5.4a, b showed that the inhibition of three metal ions tested to Cu$^{2+}$ and Ni$^{2+}$ uptake by *P. putida* 5-x was in a decreasing order of Pb$^{2+}$ > Zn$^{2+}$ > Ni$^{2+}$, and Pb$^{2+}$ > Cu$^{2+}$ > Zn$^{2+}$, respectively. 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$^{2+}$ < Zn$^{2+}$ < Cu$^{2+}$ < Pb$^{2+}$ [Hubey, 1983]. As shown by the series, both Ni$^{2+}$ and Zn$^{2+}$ form less stable complexes with electronegative ligands than Cu$^{2+}$ and Pb$^{2+}$ do. Thus the presence of Pb$^{2+}$ reduced the Cu$^{2+}$ removal capacity by 3 folds, while the presence of Cu$^{2+}$, Zn$^{2+}$ and Pb$^{2+}$ all obviously inhibited the Ni$^{2+}$ adsorption capacity. The strong inhibition might be due to its high electropositivity thus strong association with electro-negative ligands, (the electropositivity of lead is 2.33 and that of copper is 1.90) [Hubey, 1983]. In fact, Pb$^{2+}$ generally shows a very high affinity to *P. putida* 5-x. However, Pb$^{2+}$ and Zn$^{2+}$ are in trace, and both Ni$^{2+}$ and Cu$^{2+}$ are predominant in electroplating effluents of Hong Kong. Thus the effect of interaction of Ni$^{2+}$ and Cu$^{2+}$ on biosorption of *P. putida* 5-x will be further studied in detail.
The concentration of each metal ion used in this experiment was 1 mM. This value was known from previous studies to be high enough for saturating all the binding sites of *P. putida* S-x cell under the experimental conditions. Many researchers have also investigated the effects of competing co-ions on the target heavy metal removal by their biosorbents. However, the concentration of metal ions was not high enough for saturating all the binding sites on biomass, like the works done by William *et al.* (1998). If the metal ion binding sites on biosorbents have not reached saturation, no or insignificant effect on the competition of metals may be observed. Hence, misleading results would be obtained.
Figure 5.4a: Effect of Different Cations on the Copper adsorption capacity of \textit{P. putida} 5-x
Figure 5.4b: Effect of other Cations on Nickle Adsorption Capacity

- Nickle Adsorption capacity
5.5 Effect of Interaction between Cu$^{2+}$ and Ni$^{2+}$ on Adsorption of *P. putida* 5-x Cell Biomass

As mentioned above, the presence of Cu$^{2+}$ obviously inhibited Ni$^{2+}$ adsorption capacity, thus, for removal of Cu$^{2+}$ and Ni$^{2+}$ from electroplating effluent, the effect of interaction between Cu$^{2+}$ and Ni$^{2+}$ on adsorption of *P. putida* 5-x cell was studied in detail in this section. A series of adsorption experiment was carried out in wastewater containing both Cu$^{2+}$ and Ni$^{2+}$ with different ratio of molar concentrations such as 1:1, 0.6:1, 0.4:1, 0.2:1, 0.1:1 and 0.05:1. The results in Table 5.1 showed that Cu$^{2+}$ obviously impacted Ni$^{2+}$ binding on cell biomass until the ratio of Cu$^{2+}$: Ni$^{2+}$ in wastewater decreased to 0.1:1 or below.

From this results one might speculate that Ni$^{2+}$ form less stable complexes with binding site on cell surface than Cu$^{2+}$. According to the experimental results, a two-stage adsorption process should be developed to efficiently remove Cu$^{2+}$ and Ni$^{2+}$ sequentially from electroplating wastewater using *P. putida* 5-x cell biomass as biosorbent. In first stage, the cell biomass prepared as optimal biosorbent for Cu$^{2+}$ removed Cu$^{2+}$ from wastewater. When the Cu$^{2+}$ in wastewater was reduced to 2 mg L$^{-1}$ (0.03 mmol L$^{-1}$) or below, the Ni$^{2+}$ in wastewater then could be efficiently adsorbed by *P. putida* 5-x cell biomass prepared as optimal biosorbent for Ni$^{2+}$ in second stage. The major advantage of two-stage adsorption process was Ni$^{2+}$ and Cu$^{2+}$ could be recovered individually without complex separation process. The performances of the two-stage biosorption process for sequentially removing Cu$^{2+}$ and Ni$^{2+}$ from electroplating effluent are detailed in following section 7.4
Table 5.1 Effect of interaction between Cu\(^{2+}\) and Ni\(^{2+}\) in wastewater on adsorption of *P. putida* 5-x cell biomass

<table>
<thead>
<tr>
<th>Cu(^{2+}): Ni(^{2+})</th>
<th>Cu(^{2+}) after adsorption (mmol L(^{-1}))</th>
<th>Ni(^{2+}) after adsorption (mmol L(^{-1}))</th>
<th>Cu(^{2+}) removal efficiency (%)</th>
<th>Ni(^{2+}) removal efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5:0</td>
<td>0.018</td>
<td>/</td>
<td>96.4</td>
<td>/</td>
</tr>
<tr>
<td>0:0.5</td>
<td>/</td>
<td>0.030</td>
<td>/</td>
<td>93.9</td>
</tr>
<tr>
<td>0.5:0.5</td>
<td>0.020</td>
<td>0.475</td>
<td>96.0</td>
<td>5.0</td>
</tr>
<tr>
<td>0.3:0.5</td>
<td>0.014</td>
<td>0.466</td>
<td>95.3</td>
<td>6.8</td>
</tr>
<tr>
<td>0.2:0.5</td>
<td>0.016</td>
<td>0.422</td>
<td>92.1</td>
<td>15.6</td>
</tr>
<tr>
<td>0.1:0.5</td>
<td>0.012</td>
<td>0.285</td>
<td>88.2</td>
<td>43.3</td>
</tr>
<tr>
<td>0.05:0.5</td>
<td>0.011</td>
<td>0.051</td>
<td>77.8</td>
<td>89.8</td>
</tr>
<tr>
<td>0.025:0.5</td>
<td>0.008</td>
<td>0.032</td>
<td>68.1</td>
<td>93.5</td>
</tr>
</tbody>
</table>

* 1.0 g L\(^{-1}\) cell biomass was used in these adsorption experiments. All data was average value of two experimental results
5.6 Effect of Anions on Copper and Nickel Adsorption Capacity

Anions such as chloride, sulfate and nitrate are often found in industrial wastewater together with heavy metal ions. In this study, the effect of presence of chloride, nitrate and sulfate in wastewater on Cu$^{2+}$ and Ni$^{2+}$ uptake by *P. putida* 5-x cell was assessed. For all the anions, sodium ion was used as the cationic component, as it was reported in the literature that sodium ion did not influence the biosorption of copper ion [Matheickal and Yu, 1999; Zhou and Kiff, 1991]. Figure 5.5 presented the effects of various anions on Cu$^{2+}$ and Ni$^{2+}$ adsorption capacity of *P. putida* 5-x. The presence of chloride ions slightly inhibited Cu$^{2+}$ uptake, but the presence of nitrate and sulfate ion did not inhibited the Cu$^{2+}$ adsorption capacity. All three anions did not inhibit the Ni$^{2+}$ uptake. However, EDTA obviously inhibited both Cu$^{2+}$ and Ni$^{2+}$ uptake.

In general, the presence of some negative-charge ligands is usually assumed to reduce the sorption of metals to bacteria because they would contribute the formation of metal-ligand complexes, thus lowering the concentration of free metal ions in solution and showing inhibition on metals uptake [Tobin et al., 1988]. The effects of anions on biosorption correlated well with the stability constants of each cation-anion pair [Modak and Natarajan, 1995].

The present results may be explained by Pearson’s principle (i.e. hard and soft acids and bases principle). Pearson suggested that hard acids prefer to bind to hard bases and soft acids prefer to bind to soft bases [Miessier and Tarr, 1999]. Since Cu$^{2+}$ and Ni$^{2+}$ were classified as a borderline ion, the stability constants for Cu$^{2+}$ and Ni$^{2+}$ to hard anions (Cl, SO$_4^{2-}$ and NO$_3^-$) would not be high. As a result, low inhibition effect on Cu$^{2+}$ and Ni$^{2+}$. In the contrary, EDTA$^{2-}$ ligand could bind Cu$^{2+}$ and Ni$^{2+}$ to form stable chelate, thus lowering the concentration of free Cu$^{2+}$ and Ni$^{2+}$ in solution and showing inhibition on
Cu$^{2+}$ and Ni$^{2+}$ uptake by *P. putida* 5-x cell.

The studied anions commonly occurred in the electroplating effluent in Hong Kong [Chiu *et al.*, 1987], and apparently they had little effect on Cu$^{2+}$ and Ni$^{2+}$ 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 low pH HCN gas would be evolved.
Figure 5.5 Effect of anions on Copper and Nickle Adsorption Capacity

- Copper Adsorption capacity
- Nickle Adsorption Capacity

Adsorption Capacity (mg/g)

Control  Chloride  Nitrate  Sulfate  EDTA
5.7 Copper and Nickle Uptake Isotherm

Developing mathematical models on biosorption can describe the process quantitatively and aid in optimising its operating conditions. Mathematical model also can assist in reducing the number of experiments performed: the initial conditions of the experiment can be chosen more judiciously if one knows what outcome may be expected. Modelling is all the more important for applications on an industrial scale since any trials at this level are rather expensive.

The basic evaluation of sorption systems relies on the classical sorption isotherm derived from equilibrium batch contact experiments carried out under controlled environmental conditions. Quantitative comparison in metal-uptake capacity of different biosorbents can be carried out by fitting the experimental data into adsorption isotherms. Among these, single-layer adsorption isotherm models of *Langmuir* and *Freundlich*, and multi-layer adsorption isotherm model of Brunauer-Emmett-Teller (BET) are commonly used to describe the biosorption characteristics of biosorbents [Veglio' and Beolchini, 1997; Modak and Natarajan, 1995].

In this section, the Cu$^{2+}$ and Ni$^{2+}$ adsorption models by fresh cell and 0.1 mol L$^{-1}$ HCl pretreated cell were studied, while the effect of pH on adsorption model was assessed for determining optimal operation parameters for wastewater with different pH.

A series of adsorption process was carried out as procedure of section 3.4.4, and the Cu$^{2+}$ and Ni$^{2+}$ biosorption isotherms for fresh cell and prereated cell were obtained as in Figure 5.6 and 5.7. The adsorption experiments showed that the Cu$^{2+}$ adsorption processes of pretreated cells by 0.1 mol L$^{-1}$ HCl and fresh cells could be expressed as follows, respectively:

\[ Q_p = 26.9 \times C_e^{0.75} \quad \text{and} \quad Q_p = 12.8 \times C_e^{0.64} \]
This illustrated that the Cu$^{2+}$ uptake of pretreated cells and fresh cells all obeyed *Freundlich* isotherm of $Q = K_f C_e^n$. The two formulas could be linearized to following formation, respectively (see Figure 5.8).

$$\log Q_p = 1.43 + 0.75 \log C_e,$$
$$\log Q_f = 1.11 + 0.64 \log C_e,$$

Similarly, Ni$^{2+}$ adsorption processes of 0.1 mol L$^{-1}$ HCl pretreated cells and fresh cells could be expressed as

$$Q_p = 18.2 C_e^{0.27}, \text{ and } Q = 10.9 C_e^{0.265}$$

Obviously they also obeyed *Freundlich* isotherm of $Q = K_f C_e^n$. Where $C_e$ is the metal residual concentration in solution after adsorption; $Q$ is heavy metal adsorption capacity. Constant $K_f$ gives a measure of the effectiveness of Cu$^{2+}$ (or Ni$^{2+}$) biosorption at low Cu$^{2+}$ (or Ni$^{2+}$) concentration in solution. Higher value of $K_f$ means a higher biosorption level at low solution concentration. The constant $n$ measures the extent of impact on biosorption of a change in residual solution concentration from unity. High value for $n$ implies a relatively large change in sorbed Cu$^{2+}$ when the residual Cu$^{2+}$ concentration deviates either above or below unity [Weber, 1985; Sag and Kutsal, 1995]

The $K_f$ of pretreated cells and fresh cells was 26.9 and 12.9 for Cu$^{2+}$ adsorption, and 18.2 and 10.9 for Ni$^{2+}$ adsorption, respectively, indicated that pretreated cell by 0.1 mol L$^{-1}$ HCl was a better absorbent for Cu$^{2+}$ and Ni$^{2+}$ removal than fresh cell.

The *Freundlich* isotherm is an empirical expression that encompasses the heterogeneity of adsorbent surface and the exponential distribution of adsorption sites energies. Cu$^{2+}$ and Ni$^{2+}$ uptake by fresh cell and pretreated cell of *P. putida* 5-x fitted the *Freundlich* isotherm well, implying that Cu$^{2+}$ and Ni$^{2+}$ uptake by these biosorbent was largely an adsorption process. As shown by the isotherm, a residual Cu$^{2+}$ or Ni$^{2+}$ concentration in solution below 1 mg L$^{-1}$ could be attained and this showed that *P. putida* 5-x fulfilled the
requirement of good biosorbent.

According to above adsorption isotherms, if one knows the Cu$^{2+}$ or Ni$^{2+}$ concentration in wastewater, and the required discharge standard in effluent, the operation parameters, such as required adsorption capacity, amount of biosorbent in biosorption reactor would be determined.

Since biosorption can dramatically change with pH. Data are comparable only if they are obtained at the same pH. Thus biosorption models of fresh cell as biosorbent in different pH solution were studied for determining the optimal operation parameter for each pH solution.

Experimental results showed in Figure 5.9 and 5.10 that pH not only affected the constants in adsorption models for both Cu$^{2+}$ and Ni$^{2+}$, but the types of adsorption isotherm seemed to be also different. In higher pH solution (pH 6.5 and 8.0), adsorption for Cu$^{2+}$ and Ni$^{2+}$ by fresh cell obeyed the *Freundlich* models of

$$Q_{Cu} = 74.2 \, Ce^{0.39} \quad \text{and} \quad Q_{Cu} = 12.8 \, Ce^{0.64}$$

$$Q_{Ni} = 17.6 \, Ce^{0.365} \quad \text{and} \quad Q_{N} = 10.9 \, Ce^{0.265}$$

respectively. However, in low pH solution (pH 4.5), the adsorption process seemed to be better described by equations of

$$Ce / Q_{Cu} = 0.0774 + Ce / 37.8 \quad \text{for} \quad Cu^{2+}$$

and

$$Ce / Q_{Ni} = 0.1 + Ce / 20.2 \quad \text{for} \quad Ni^{2+},$$

respectively, obviously they obeyed *Langmuir* isotherm of \( Ce / q = 1 / (q_{max} \, b) + Ce / q_{max} \). Where \( q_{max} \) represents the saturation level of sorbed Cu$^{2+}$ or Ni$^{2+}$ at high solution concentrations, and \( b \) is the *Langmuir* constant, a ratio of the adsorption rate constant to the desorption rate constant [Volesky, 1994]. From the two equations, we could find that
in pH 4.5 solution, the maximum adsorption capacity to Cu$^{2+}$ and Ni$^{2+}$ were 37.8 mg g$^{-1}$ and 20.2 mg g$^{-1}$, respectively, which were obviously lower adsorption levels.

The results indicated that adsorption models, hence adsorption properties, in pH 4.5 solution differed from in pH 6.5 and pH 8.0 solution, and the fresh cell as biosorbent in pH 8.0 solution had a higher biosorption level than in pH 6.5 solution due to the K$_f$ in pH 8.0 solution much more large than in pH 6.5 solution. In wastewaters with different pH, different adsorption models should be used to determine the adsorption parameters.

Adsorption isotherms are useful in the description of adsorption behaviour of particular adsorbate-adsorbent systems over a range of equilibrium concentration of the adsorbate at a particular pH. However, the relation of removal capacity and equilibrium concentration of Cu$^{2+}$ or Ni$^{2+}$ 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. Above adsorption isotherms were obtained just in the single metal ion system, the effects of other cations or anions on adsorption isotherms of Cu$^{2+}$ and Ni$^{2+}$ uptake were more complex and were not considered in this project.
Figure 5.6 Cu²⁺ adsorption isotherms of fresh cell and pretreated cell of *P. putida* 5-x
**Figure 5.7** Ni$^{2+}$ adsorption isotherms of fresh cell and pretreated cell of *P. putida* 5-x
Figure 5.8 Linearized Cu\(^{2+}\) adsorption isotherms of fresh cell and pretreated cell of *P. putida* 5-x.
Figure 5.9. Copper Uptake Isotherms in Different pH Solution

\[ Q = 74.2 \, \text{Ce}^{0.39} \]

\[ Q = 12.8 \, \text{Ce}^{0.64} \]

\[ \text{Ce/Q} = 0.0774 \]

Ce of Copper (mg/L)

Adsorption Capacity (mg/g)
Figure 5.10. Nickel Uptake Isotherm in Different pH Solution

\[ Q = 17.6 \, Ce^{0.365} \]

\[ Q = 10.9 \, Ce^{0.265} \]

\[ Ce/Q = 0.1 + Ce/20.2 \]
Chapter 6: Copper and Nickel Desorption and Biosorbent Regeneration and Reuse

The use of bacterial biomass as a potential biosorbent depends not only on the biosorptive capacity, but also on how well the biomass can be regenerated and reused. In industrial applications of biosorption, the adsorbed metal ions on biosorbent need to be recovered in a concentrated solution, simultaneously regenerating the biosorbent for reuse for economical reasons.

The optimal desorption technique for the recovering absorbed metal ions from the cell biomass and the regeneration of biosorbent were investigated in this studied. The attractions focused on cell non-destructive desorption techniques due to considering of cell regeneration and reuse. In addition, the desorption properties and desorption kinetics of *P. putida* 5-x cell biomass were studied.

6.1 Desorption Eluants

Ten solutions similar to the eluants used in cell pretreatment (see Table 6.1) were chosen as desorption solution to test their Cu$^{2+}$ and Ni$^{2+}$ desorption rate from loaded biosorbent. Ammonium and sodium acted as competing cations are considered as exchangers to replace the Cu$^{2+}$ and Ni$^{2+}$ bound on binding site of biosorbent. The mineral acids (H$_2$SO$_4$, HCl) are considered to be protons exchanging agents. The availability of different desorption solution depended on their desorption rates and the biomass loss rates during the course of desorption. A better desorption solution should have high Cu$^{2+}$ and Ni$^{2+}$ desorption rate and low biomass loss rate. Table 6.1 listed the performances of different solution in desorbing Cu$^{2+}$ and Ni$^{2+}$ from loaded *P. putida* 5-x cell biomass. It
clearly showed that the mineral acids appeared higher Cu$^{2+}$ and Ni$^{2+}$ desorption rate (>96 % and >98 %, respectively) than sodium amonium salt and alkli. It is because that sodium and ammonium ion just acted as competing counter-ions to replace Cu$^{2+}$ and Ni$^{2+}$ from binding site on biosorbent. However, the H$^+$ of mineral acids not only acted as proton to exchange Cu$^{2+}$ and Ni$^{2+}$ from binding site, but also decreased the pH of solution, thus change some electronegative binding groups in higher pH such as hydroxyl, sulphhydryl, thioether and amine on cell surface to electropositive, therefore declined the affinity between heavy metal ions and cell surface, hence enhanced the desorption rate of Cu$^{2+}$ and Ni$^{2+}$ from biomass [Schiewer and Volesky, 2000]. This illustrated that the desorbing power was not only depended on the competitive abilities between the target metal ions and other cations presented to the active sites on the biomass surface, but also on the solution pH.

From Table 6.1, it could be also find that, diluted mineral acids such as 0.1mol L$^{-1}$ HCl showed a quite low BLR than concentrated mineral acids, although slightly low desorption rate. Thus, considering to both desorption rate and BLR, 0.1~0.2 mol L$^{-1}$ HCl was a better desorption solution for recovery of Cu$^{2+}$ and Ni$^{2+}$ from loaded biosorbent and simultaneous cell biomass regeneration. In addition, 0.1~0.3 mol L$^{-1}$ HCl was also considered as better cell pretreating eluant on above study. The unification of the cell pretreating solution and desorption/regeneration solution could simplify the operation and save cost in practical application.
<table>
<thead>
<tr>
<th>Desorption Solution</th>
<th>Cu$^{2+}$ Desorption Rate (%)</th>
<th>Ni$^{2+}$ Desorption Rate (%)</th>
<th>BLR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mol L$^{-1}$ HCl</td>
<td>96.1</td>
<td>99.1</td>
<td>3.1</td>
</tr>
<tr>
<td>0.3 mol L$^{-1}$ HCl</td>
<td>96.7</td>
<td>99.3</td>
<td>6.3</td>
</tr>
<tr>
<td>0.6 mol L$^{-1}$ HCl</td>
<td>96.8</td>
<td>99.2</td>
<td>9.1</td>
</tr>
<tr>
<td>0.1 mol L$^{-1}$ H$_2$SO$_4$</td>
<td>96.6</td>
<td>98.7</td>
<td>8.3</td>
</tr>
<tr>
<td>0.3 mol L$^{-1}$ H$_2$SO$_4$</td>
<td>96.9</td>
<td>98.9</td>
<td>8.9</td>
</tr>
<tr>
<td>0.6 mol L$^{-1}$ H$_2$SO$_4$</td>
<td>97.2</td>
<td>99.1</td>
<td>11.2</td>
</tr>
<tr>
<td>10% (NH$_4$)$_2$SO$_4$</td>
<td>54.9</td>
<td>59.9</td>
<td>2.3</td>
</tr>
<tr>
<td>15% (NH$_4$)$_2$SO$_4$</td>
<td>62.8</td>
<td>71.2</td>
<td>2.9</td>
</tr>
<tr>
<td>0.1 mol NaOH</td>
<td>2.9</td>
<td>21.2</td>
<td>16.6</td>
</tr>
<tr>
<td>0.3 mol NaOH</td>
<td>2.8</td>
<td>18.9</td>
<td>19.8</td>
</tr>
</tbody>
</table>

* Desorption rates were determined after desorption 10 min of 100mg saturated biosorbent by 5 ml different desorption solution All data was average value of three experimental results
6.2 Effect of Ratio of Desorption Solution Volume to Biosorbent Mass on Copper and Nickle Desorption

Desorbing solution may have dual functions. Not only can they be used to desorb bound metal from the biosorbents and to regenerate the biomass for the next biosorption operation, but also to provide an eluate from which metal can be recovered directly by electrolysis or precipitation [Butter et al. 1998]. For second objective, not only desorption rate, but also concentration of $\text{Cu}^{2+}$ and $\text{Ni}^{2+}$ in desorbed solution should be considered. In this study, the optimal ratio of desorption solution volume to biosorbent mass was investigated. After adsorption process, 100 mg biomass saturated by $\text{Cu}^{2+}$ or $\text{Ni}^{2+}$ were desorbed by 0.1 mol L$^{-1}$ HCl with different volumes. Experimental results showed in Table 6.2 that $\text{Cu}^{2+}$ or $\text{Ni}^{2+}$ in recovered solution was quite high while desorption rate was 90% below when the volume of desorption solution was less. However, more than 96% and 99% recovery efficiency were obtained by using 5 ml or more of 0.1 mol L$^{-1}$ HCl, while the $\text{Cu}^{2+}$ and $\text{Ni}^{2+}$ concentration in the recovery solution was 1.62 g L$^{-1}$ and 0.75 g L$^{-1}$ below, respectively. Experimental results showed when volume of desorption solution exceeded 5 ml, the desorption rate reached the top in general, but the less the volume of desorbing solution used, the more the concentration of metal could be obtained in desorption solution, thus the higher the cost efficiency. As a result, the ratio of 5 ml desorbing solution to 100 mg loaded biomass was used in the following desorption experiment studies. The concentration of 1.62 g L$^{-1}$ Cu$^{2+}$ or 0.75 g L$^{-1}$ Ni$^{2+}$ in the desorbed solution will be proved to be high enough for further recovery by electrolysis or precipitation.
<table>
<thead>
<tr>
<th>0.1mol HCl Volume (mL)</th>
<th>1.0</th>
<th>2.5</th>
<th>5.0</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu(^{2+}) Concentration (g L(^{-1}))</td>
<td>6.11</td>
<td>2.81</td>
<td>1.63</td>
<td>0.82</td>
<td>0.55</td>
</tr>
<tr>
<td>Ni(^{2+}) Concentration (g L(^{-1}))</td>
<td>3.17</td>
<td>1.39</td>
<td>0.75</td>
<td>0.38</td>
<td>0.26</td>
</tr>
<tr>
<td>Cu(^{2+}) Desorption Rate (%)</td>
<td>72.4</td>
<td>83.1</td>
<td>96.1</td>
<td>96.8</td>
<td>97.1</td>
</tr>
<tr>
<td>Ni(^{2+}) Desorption Rate (%)</td>
<td>83.2</td>
<td>91.1</td>
<td>99.2</td>
<td>99.7</td>
<td>99.8</td>
</tr>
</tbody>
</table>
6.3 Copper and Nickel Desorption Kinetics

Since diluted HCl may also cause cell conformation change, thus result in the structure damage and biomass loss if the desorbing time is too long. The bare contact time between biomass and the acid in desorption is critical for the biomass reuse potential. The shorter the contact times, the low the biomass loss rate. In order to optimize the metal recovery conditions, experiment was conducted to investigate the efficiency of 0.1 mol L\(^{-1}\) HCl for desorption of Cu\(^{2+}\) and Ni\(^{2+}\) from \textit{P. putida} 5-x cell.

The kinetics of Cu\(^{2+}\) and Ni\(^{2+}\) desorption from the loaded biomass was demonstrated in Figure 6.1, while the cell biomass loss rate along with desorption time was assessed. It could clearly be found that the desotpyion of Cu\(^{2+}\) and Ni\(^{2+}\) was very rapid, however, within first 2.5 min, more than 95 % of Ni\(^{2+}\) could be desorbed while only less than 90 % Cu\(^{2+}\) was desorbed. After desorbing 5 min, 99 % of Ni\(^{2+}\) was desorbed from loaded biosorbent, while 96 % of Cu\(^{2+}\) was desorbed after 10 min. This illustrated that, although both Cu\(^{2+}\) and Ni\(^{2+}\) could be desorbed efficiently and quickly by diluted HCl, Ni\(^{2+}\) was more easily to be desorbed by diluted HCl. The Cu\(^{2+}\) and Ni\(^{2+}\) desorption reached real equilibrium at about 45 min and 30 min, respectively. The result was comparable to that obtained by Chang \textit{et al.} (1997). They found that desorption equilibrium was nearly reached shortly after the metal-laden \textit{Pseudomonas aeruginosa} PU21 was in contact with 0.1 mol L\(^{-1}\) HCl for 5 min, but really reached equilibrium for 30~45 min. In Figure 6.1, it was also clearly found that, along with desorption times by 0.1 mol L\(^{-1}\) HCl, cell biomass loss rate increased obviously from 1.7 % of 2.5 min to 7.2 % of 45 min. Although, in real equilibrium phase, Cu\(^{2+}\) and Ni\(^{2+}\) desorption rate was slight higher (97.3 % and 99.5 %, respectively) than in near equilibrium phase, the biomass loss rate was much higher than in near equilibrium. Considering to both BLR and desorption rate, contacting 5~10 min between 0.1 mol L\(^{-1}\) HCl and loaded cell biomass was an optimal desorption time for
recovery of Cu²⁻ and Ni²⁻ from biosorbent, and simultaneously for cell regeneration.
Figure (6.1) Time Curve of Copper and Nickle Desorption and Biomass Loss Rate

- ▲ Copper Desorption Rate (%)
- ■ Nickle Desorption Rate (%)
- ○ Biomass Loss Rate (%)

Desorption Time (%) vs. Desorption Times

0 10 20 30 40 50 60 70
0 20 40 60 80 100 120

0 1 2 3 4 5 6 7 8

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6.4. Cell Regeneration and Reuse

Effective reuse of regenerated cell for adsorption and recovery of heavy metal is critical for industrial application at low cost. The more the cell reuse times, the less the biosorbent consumption, thus the less cost for biosorbent disposal and transport, hence the less total cost. The cell reusability depended on the effective desorption and cell regeneration technique. The appearances of cell biomass loss rate, heavy metal adsorption capacity and desorption rate in adsorption/desorption cycle decided whether could the cell biomass be reused many times. As above experiment showed that diluted mineral acid effectively desorbed Cu\(^{2+}\) and Ni\(^{2+}\) from loaded biomass, and simultaneously regenerated cell biomass with low biomass loss rate. Repeated biosorption/desorption operation cycle using different diluted mineral acid, such as 0.1 mol L\(^{-1}\), 0.3 mol L\(^{-1}\) HCl and 0.1 mol L\(^{-1}\) H\(_2\)SO\(_4\) as desorption (regeneration) solution were carried out in this section to examine the reusability of P. putida 5-x cell, such as Cu\(^{2+}\) and Ni\(^{2+}\) adsorption/desorption efficiency and biomass loss rate in every adsorption/desorption cycle.

Five consecutive Cu\(^{2+}\) and Ni\(^{2+}\) biosorption/desorption (regeneration) cycles were conducted according to the procedure of section 3.4.1 and 3.5.1, and the results were plotted in Figure 6.2 and Figure 6.3. For all selected diluted mineral acids as desorption and cell regeneration reagent, the adsorption capacity and desorption rate of both Cu\(^{2+}\) and Ni\(^{2+}\) did not change obviously from cycle 1 to cycle 5. For 0.1 mol L\(^{-1}\) HCl, more than 84 mg g\(^{-1}\) Cu\(^{2+}\) and 37 mg g\(^{-1}\) Ni\(^{2+}\) could be adsorbed by regenerated cell in all runs, and more than 96 % of sorbed Cu\(^{2+}\) and 99 % sorbed Ni\(^{2+}\) could be recovered. The cell biomass loss rate in each cycle was 2 % below, exception of cycle 1. For 0.3 mol L\(^{-1}\) HCl and 0.1 mol L\(^{-1}\) H\(_2\)SO\(_4\), slightly low Cu\(^{2+}\) and Ni\(^{2+}\) adsorption capacity and slightly high desorption rate could be obtained in each cycle, in general trends, compared with 0.1 mol
L· HCl as regeneration solution, but the biomass loss rate in each cycle was obviously higher than 0.1 mol L· HCl as desorption solution. This result indicated that P. putida 5-x cell possessed high reuse potential, at least five cycles, for removal and recovery of Cu²⁺ and Ni²⁺ from wastewater. Among the mineral acids, 0.1 mol L· HCl was of particular advantage for desorping heavy metal and regenerating cell biomass due to its low BLRs and higher adsorption capacity during the desorption and regeneration course.

In additional, it was interested to find that Cu²⁺ and Ni²⁺ uptake slightly increased in the second uptake-cycle, and Cu²⁺ and Ni²⁺ desorption rate in some later cycles was slightly higher than in first cycle, the Ni²⁺ desorption rate even exceeded 100 % in cycle 3 by 0.1 mol L· HCl and cycle 2 and 5 by 0.3 mol L· HCl. The increment of Cu²⁺ or Ni²⁺ uptake in cycle 2 may be attributed to the first treatment of the desorbing solution. After desorption by mineral acid, the cell surface structure may be changed to further liberate more binding sites heavy metal assessable. Thus, the increment in binding sites on cell surface of biomass might be a reason of the increment in adsorption capacity. Similar observations were reported by Wong et al. (1993b) and Chang et al. (1997). The increment of desorption rate in later cycles might be due to some Cu²⁺ or Ni²⁺ undesorbed from cell biomass in early cycles. the remained Cu²⁺ and Ni²⁺ on cell biomass might be redesorbed in the later cycles.
Figure 6.2. Operation Results of Five Cycles Copper Adsorption/Desorption by *P. putida* 5-x cell

DR--- Desorption Rate (%)

AC--- Adsorption Capacity (mg g⁻¹)

BLR--- Biomass Loss Rate (%)

Cycles
Figure 6.3. Operation Results of Five Cycles of Nickel Adsorption/Desorption by *P. putida* 5-x Cell

DR --- Desorption Rate (%)

AC --- Adsorption Capacity (mg g⁻¹)

BLR --- Biomass Loss Rate (%)
Chapter 7: Removal and Recovery of Copper and Nickel from Wastewater by Immobilized Cells

The high surface area to volume ratio of microbial cells allows a better contact between the biosorbent surface and the metal ions in solution [Butter et al, 1996; Eccles, 1995; Simmons et al, 1995]. On the other hand, microbial biomass in its natural form consists of small particles of low density, low mechanical strength, and low rigidity. The use of such particles as biosorbent in any conventional unit operation for adsorption of metal ions from large volume of containing metal solution has been shown to not be practical. The main difficulty lies in the rapid and efficient separation of biomass from the reaction mixture after contact [Treen, 1981; Brierley et al., 1986; Mellis, 1986; Krambeer, 1987; Tsezos et al., 1987]. Alternatively, immobilized microbial biomass could be produced in the form of particles of desirable size, mechanical strength, and rigidity while maintaining the native properties of the biomass. Thus it can improve their performance in bioreactors as well as enhancing reusability [Volesky, 1987].

All in all, immobilization processes, which convert microbial biomass to a particulate form, can overcome the deficiencies associated with freely suspended cells. However, properties such as mechanical strength, chemical stability, biocompatibility and permeability for soluble metallic ions and molecules (controlled by size and porosity) of the immobilization matrix are of crucial importance for applying immobilized microbial cells in large-scale continuous bioconversion processes.

Calcium alginate, polyacrylamide and polyvinyl alcohol (PVA) are commonly used as cells immobilization matrices. However, due to less strength and permeability, the calcium alginate, polyacrylamide and polyvinyl alcohol seemed to not be reused many times [Wong et al. 1993b]. In addition, the immobilization course using calcium alginate,
polyacrylamide and polyvinyl alcohol as medium was more complex and high cost [Wong, 2001]. In this study, a novel immobilization medium-Magnetite was used for immobilizing *P. putida* 5-x cell. The magnetite-immobilization bacterial cells have four distinct advantages: (1) the separation of the immobilized cells from the treated metal-laden effluent is efficient and convenient with aids of electromagnet; (2) the material properties of the biosorbent may be manipulated with immobilization methods; (3) the cost of magnetite is low and the immobilization course is simply; and (4) the permeability is quite high due to the cells were not embedded in magnetite, just attached on magnetite. The macroscopic immobilized cells are more easily retained in a bioreactor operated in a continuous-flow mode, and pipeline blockage by the free-suspending microscopic microbial cell can avoided.

### 7.1 Copper and Nickel Uptake by Magnetite Immobilized *P. putida* 5-x Cell

The *P. putida* 5-x cell was immobilized on magnetite according to procedure of section 3.3.5, and the Cu\(^{2+}\) and Ni\(^{2+}\) uptake was studied. In the course of adsorption, the concentrations of magnetite alone and the magnetite-immobilized cell were kept at 2.5 g L\(^{-1}\) and 3 g L\(^{-1}\), respectively, in solution with different Cu\(^{2+}\) and Ni\(^{2+}\) concentrations. Figure 7.1 and 7.2 illustrated the Cu\(^{2+}\) and Ni\(^{2+}\) biosorption isotherms for magnetite alone and magnetite immobilized *P. putida* 5-x cell at pH 6.5. The results demonstrated that the magnetite alone also exhibited biosorption behavior, but magnetite immobilized *P. putida* 5-x cell appeared obviously better Cu\(^{2+}\) and Ni\(^{2+}\) adsorption behavior than magnetite alone. The uptake of magnetite alone and magnetite immobilized cell for Cu\(^{2+}\) and Ni\(^{2+}\) could be expressed as
\[ Q_{\text{M-Cu}} = 2.1 C_{\text{e-Cu}}^{0.68} \quad \text{and} \quad Q_{\text{M-Ni}} = 11.9 C_{\text{e-Cu}}^{0.74} \]
\[ Q_{\text{M-Ni}} = 0.78 C_{\text{e-Ni}}^{0.79} \quad \text{and} \quad Q_{\text{M-Ni}} = 9.7 C_{\text{e-Ni}}^{0.21} \]

respectively. Obviously they all obeyed the Freundlich isotherms of \( Q = K C^n \). The \( K \) is an indication of adsorption capacity and \( n \) indicated the effect of metal ion concentration on the adsorption capacity. The big the \( K \) value, the large the adsorption capacity; the small the \( n \), the large the effect of metal ion concentration on adsorption capacity [Chua et al. 1998]. From the isotherms, it was clearly found that magnetite immobilized cell appeared much higher adsorption capacity than magnetite alone due to \( K \) value of magnetite immobilized cell is much bigger than magnetite alone not only for \( \text{Cu}^{2+} \) but also for \( \text{Ni}^{2+} \) adsorption. The \( \text{Cu}^{2+} \) adsorption capacity of magnetite immobilized cell was higher than \( \text{Ni}^{2+} \) adsorption capacity. In addition, it was found that the effect of \( \text{Ni}^{2+} \) concentration in wastewater on \( \text{Ni}^{2+} \) adsorption capacity by magnetite immobilized cell was more obvious than by magnetite alone due to \( n \) value of magnetite immobilized cell was much smaller than that of magnetite alone. Adsorption experiment showed in low concentration of \( \text{Cu}^{2+} \) and \( \text{Ni}^{2+} \) (10 mg L\(^{-1}\) below), the difference of adsorption capacity between the magnetite immobilized cell and magnetite alone was slight, that was 3.38 mg g\(^{-1}\) and 3.2 mg g\(^{-1}\) for \( \text{Cu}^{2+} \), and 3.3 mg g\(^{-1}\) and 2.4 mg g\(^{-1}\) for \( \text{Ni}^{2+} \), respectively. On the contrary, a large difference in \( \text{Cu}^{2+} \) (or \( \text{Ni}^{2+} \)) removal capacity between the magnetite immobilized cell and magnetite alone was observed when the concentration of \( \text{Cu}^{2+} \) and \( \text{Ni}^{2+} \) in solution was about 50 mg L\(^{-1}\), that was 16.5 mg g\(^{-1}\) and 14.1 mg g\(^{-1}\) for \( \text{Cu}^{2+} \), and 14.4 mg g\(^{-1}\) and 9.5 mg g\(^{-1}\) for \( \text{Ni}^{2+} \), respectively. This was because that \( \text{Cu}^{2+} \) and \( \text{Ni}^{2+} \) were mainly taken up by support matrix at low concentration, but when the concentrations of \( \text{Cu}^{2+} \) and \( \text{Ni}^{2+} \) in solution increased to enough, the surface of the magnetite would become saturated by the metal ions, and the metal ions could diffuse into cells dispersed on magnetite.
In this study, the uptake isotherms were investigated among 10 mg L\(^{-1}\) to 100 mg L\(^{-1}\) for Cu\(^{2+}\) and 10 mg L\(^{-1}\) to 50 mg L\(^{-1}\) for Ni\(^{2+}\), but the adsorption capacities did not leveled off even at Cu\(^{2+}\) concentration of 100 mg L\(^{-1}\) and Ni\(^{2+}\) concentration of 50 mg L\(^{-1}\) in wastewater. This indicated that the adsorption isotherms in Figure 7.1 and 7.2 seemed to be applicable to more high concentration of Cu\(^{2+}\) and Ni\(^{2+}\). The contribution of P. putida 5-x cell to Cu\(^{2+}\) and Ni\(^{2+}\) adsorption by the immobilized cell could be estimated by comparing the removal capacities (Q) of the magnetite alone and magnetite immobilized cell. In addition, it was found that the Cu\(^{2+}\) and Ni\(^{2+}\) adsorption capacity of the magnetite immobilized cells was obviously lower than that of pure P. putida 5-x. This is because the adsorption capacity of magnetite was significantly low than P. putida 5-x cell, thus, the average value of adsorption capacity of magnetite immobilized cell was certainly low than that of pure P. putida 5-x cell.
Figure 7.1: Copper Adsorption Isotherm of Magnetite and Magnetite Immobilized Cell

- $Q_{\text{M-Cu}} = 11.9 \times C_{\text{e-Cu}}^{0.74}$
- $Q_{\text{M-Cu}} = 2.1 \times C_{\text{e-Cu}}^{0.68}$

Legend:
- Magnetite Alone
- Magnetite Immobilized Cell

Copper Concentration (mg/L) vs. Adsorption Capacity (mg/g)
Figure 7.2: Nickel Adsorption Isotherm of Magnetite and Immobilized Magnetite Cell

\[ Q_{\text{M-Ni}} = 9.7 C_{\text{c-Ni}}^{0.21} \]

\[ Q_{\text{Im-Ni}} = 0.78 C_{\text{c-Ni}}^{0.79} \]

- Magnetite Alone
- Magnetite Immobilized Cell
7.2 Copper (or Nickle) Removal and Recovery from Copper (or Nickle) Containing Solution Using a Semi-Continuous Biosorption Process (Stirred Reactor)

A semi-continuous stirred reactor (Figure 3.2) with magnetite-immobilized *P. putida* 5-x cell as biosorbent was used to remove and recover Cu$^{2+}$ (or Ni$^{2+}$) from Cu$^{2+}$ (or Ni$^{2+}$) containing wastewater. The Cu$^{2+}$ (or Ni$^{2+}$) bearing wastewater was fed counter-currently to the biosorbent (immobilized *P. putida* 5-x cell biomass) into the reactor in order to obtain the lowest possible Cu$^{2+}$ (or Ni$^{2+}$) concentration in effluent and highest possible Cu$^{2+}$ (or Ni$^{2+}$) concentration in the biosorbent. In adsorption, the heavy metal containing wastewater was well stirredly reacted with biosorbent in each reactor for 20 min, and settling 20 min with aid of electromagnet. The supernatant of R1 then was fed into R2 for second step adsorption, and then into R3 for third step adsorption. The supernatant of R3 finally was discharged with meeting discharge standard of Hong Kong. After adsorption and settling, the Cu$^{2+}$ (or Ni$^{2+}$) bound biosorbents in R3 and R2 were transformed to R2 and R1, respectively, for further adsorbing Cu$^{2+}$ (or Ni$^{2+}$) until to saturation. In desorption, the saturated biosorbent in R1 was passed through a 0.4μ filter and then bound Cu$^{2+}$ (or Ni$^{2+}$) was recovered, and biosorbent was regenerated simultaneously in acid regeneration reactor according to procedure of section 3.5.1. Regenerated biosorbent was returned into R3 for reuse after being washed by MES buffer in washing tank. Due to Cu$^{2+}$ and Ni$^{2+}$ concentration in electroplating effluent of Hong Kong was about 20~30 mg L$^{-1}$, the Cu$^{2+}$ (or Ni$^{2+}$) concentration in synthetic wastewater was prepared as about 30 mg L$^{-1}$. The immobilized cell concentration was 3 g L$^{-1}$. Another semi-continuous stirred reactor containing 2.5 g L$^{-1}$ magnetite alone was parallelly run as control.

After the system reached steady state, five adsorption-desorption(regeneration) cycles
were assessed. The results in Table 7.1 (or Table 7.2) showed the operation results of Cu\(^{2+}\) (or Ni\(^{2+}\)) removal and recovery in each adsorption/desorption cycle. It was found that the Cu\(^{2+}\) removal efficiency of all cycles reached 97 % above (average value was 97.46 %) treated by magnetite immobilized *P. putida* 5-x cell, while magnetite alone was only about 70 % (average value was 70.2 %). For Ni\(^{2+}\), the removal efficiency of the immobilized cell also was 97 % above (average value was 97.18 %), while magnetite alone was about 50 % (average value was 49.1 %). This indicated that the *P. putida* 5-x cell immobilized on magnetite played a more part in magnetite immobilized cell for removal of Cu\(^{2+}\) (or Ni\(^{2+}\)) from wastewater. The difference of treatment efficiency between treated by magnetite immobilized cell and magnetite alone was equivalent to the contribution of *P. putida* 5-x cell immobilized on magnetite to Cu\(^{2+}\) and Ni\(^{2+}\) removal. It was clear from Table 7.1 and Table 7.2 that about 30 % Cu\(^{2+}\) (or 50 % Ni\(^{2+}\)) was removed by 0.5 g L\(^{-1}\) (1/6 of total biosorbent) *P. putida* 5-x cell immobilized on magnetite. However, from these results, we could find that the amount of Cu\(^{2+}\) bound by *P. putida* 5-x cell immobilized on magnetite was low to amount of Ni\(^{2+}\), this conflict with above experimental results. The reason is the concentration of Ni\(^{2+}\) in that case acted on *P. putida* 5-x cell was much higher than that of Cu\(^{2+}\) due to adsorption capacity of magnetite alone to Cu\(^{2+}\) was higher than to Ni\(^{2+}\). Therefore the cell biomass was not saturated by far with Cu\(^{2+}\) in this low concentration large of Cu\(^{2+}\) acted on the magnetite powder, and only small Cu\(^{2+}\) acted on *P. putida* 5-x cell attached on magnetite. The desorption rate of Cu\(^{2+}\) from loaded immobilized cell in each adsorption/desorption cycle was 96 % above, (for Ni\(^{2+}\) was 98 % above), and the Cu\(^{2+}\) concentration in desorption solution was 0.71 g L\(^{-1}\) above (For Ni\(^{2+}\) was 0.72 g L\(^{-1}\) above). This illustrated that the 0.1 mol L\(^{-1}\) HCl was efficient to desorb Cu\(^{2+}\) (or Ni\(^{2+}\)) from loaded biosorbent, and the concentration of recovered Cu\(^{2+}\) (or Ni\(^{2+}\)) in desorbed solution was enough high for further recovery by
electrolysis or precipitation [Krishnan et al. 1993]. These experimental results indicated that the Semi-Continuous Stirred Reactor with magnetite immobilized *P. putida S*-x cell as biosorbent was efficient to remove and recover Cu$^{2+}$ (or Ni$^{2+}$) from Cu$^{2+}$ (or Ni$^{2+}$) containing wastewater. The magnetite immobilized cell could be reused efficiently five cycles at least.

The section just considered the situation of wastewater only containing one metal ion (Cu$^{2+}$ or Ni$^{2+}$), but in practical application, Cu$^{2+}$ and Ni$^{2+}$ are simultaneously and largely present in the electroplating effluent. Thus the biosorption process for removal and recovery of Cu$^{2+}$ and Ni$^{2+}$ sequentially from both Cu$^{2+}$ and Ni$^{2+}$ containing should be further studied.
Table 7.1 Cu$^{2+}$ removal and recovery using semi-continuous stirred reactor with magnetite immobilized P. putida 5-x cell as biosorbent*

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Initial Cu$^{2+}$ (mg L$^{-1}$)</th>
<th>Final Cu$^{2+}$ (mg L$^{-1}$)</th>
<th>Removal Efficiency (%)</th>
<th>Cu$^{2+}$ in Desorbed Solution (g L$^{-1}$)</th>
<th>Desorption Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30.5</td>
<td>0.72(9.1)</td>
<td>97.6(69.9)</td>
<td>0.716</td>
<td>96.1</td>
</tr>
<tr>
<td>2</td>
<td>30.7</td>
<td>0.67(8.7)</td>
<td>97.8(71.3)</td>
<td>0.724</td>
<td>96.4</td>
</tr>
<tr>
<td>3</td>
<td>30.4</td>
<td>0.73(9.3)</td>
<td>97.6(69.5)</td>
<td>0.72</td>
<td>97.1</td>
</tr>
<tr>
<td>4</td>
<td>30.2</td>
<td>0.77(8.8)</td>
<td>97.5(70.9)</td>
<td>0.713</td>
<td>96.9</td>
</tr>
<tr>
<td>5</td>
<td>30.4</td>
<td>0.78(9.0)</td>
<td>97.4(70.1)</td>
<td>0.712</td>
<td>96.2</td>
</tr>
<tr>
<td>Average</td>
<td>30.4</td>
<td>0.734(9.0)</td>
<td>97.46(70.2)</td>
<td>0.717</td>
<td>96.26</td>
</tr>
</tbody>
</table>

* The values in bracket came from control reactor. Biosorbent concentration was 3 g L$^{-1}$ in real reactor and 2.5 g L$^{-1}$ in control reactor. The ratio of desorption solution to biosorbent was 40 ml to 3 g biosorbent.
Table 7.2 Ni\textsuperscript{2+} removal and recovery using semi-continuous stirred reactor with magnetite immobilized *P. putida* 5-x cell as biosorbent*

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Initial Ni\textsuperscript{2+} (mg L\textsuperscript{-1})</th>
<th>Final Ni\textsuperscript{2+} (mg L\textsuperscript{-1})</th>
<th>Removal Efficiency (%)</th>
<th>Ni\textsuperscript{2+} in Desorbed Solution (g L\textsuperscript{-1})</th>
<th>Desorption Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30.2</td>
<td>0.84 (15.1)</td>
<td>97.2 (50.5)</td>
<td>0.728</td>
<td>99.1</td>
</tr>
<tr>
<td>2</td>
<td>30.3</td>
<td>0.87 (14.8)</td>
<td>97.4 (51.8)</td>
<td>0.731</td>
<td>99.4</td>
</tr>
<tr>
<td>3</td>
<td>30.5</td>
<td>0.88 (15.7)</td>
<td>97.1 (48.3)</td>
<td>0.738</td>
<td>99.7</td>
</tr>
<tr>
<td>4</td>
<td>30.2</td>
<td>0.87 (16.1)</td>
<td>97.1 (46.7)</td>
<td>0.725</td>
<td>98.9</td>
</tr>
<tr>
<td>5</td>
<td>30.1</td>
<td>0.87 (15.6)</td>
<td>97.1 (48.7)</td>
<td>0.725</td>
<td>99.2</td>
</tr>
<tr>
<td>Average</td>
<td>30.26</td>
<td>0.866 (15.5)</td>
<td>97.18 (49.1)</td>
<td>0.729</td>
<td>99.26</td>
</tr>
</tbody>
</table>

* The values in bracket came from control reactor. Biosorbent concentration was 3 g L\textsuperscript{-1} in real reactor and 2.5 g L\textsuperscript{-1} in control reactor. The ratio of desorption solution to biosorbent was 40 ml to 3 g biosorbent.
7.3 Sequential Removal and Recovery of Copper and Nickel from Synthetic Wastewater Using Two-Stage Biosorption Process

Cu$^{2+}$ and Ni$^{2+}$ are main heavy metal ions in electroplating effluent of Hong Kong. Although the magnetite immobilized cell was efficient to adsorb Cu$^{2+}$ (or Ni$^{2+}$) from single metal ion containing solution, the experimental result in Section 5.5 showed that Cu$^{2+}$ present in solution obviously inhibited the Ni$^{2+}$ binding by P. putida 5-x cell, while Ni$^{2+}$ hardly inhibited the Cu$^{2+}$ binding. For effectively removing and recovering Cu$^{2+}$ and Ni$^{2+}$ from both Cu$^{2+}$ and Ni$^{2+}$ containing wastewater, simultaneously, according to adsorption properties of Cu$^{2+}$ and Ni$^{2+}$ by P. putida 5-x cell, a two stages biosorption process was developed to sequentially removal and recovery of Cu$^{2+}$ and Ni$^{2+}$ from wastewater. The two stages biosorption process was made of two series linked semi-continuous stirred reactor (Figure 3.2) with magnetite immobilized P. putida 5-x cell as biosorbent. In stage 1, the P. putida 5-x cell biomass as biosorbent was prepared with optimal production condition for Cu$^{2+}$ adsorption, and in stage 2, the cell biomass was produced with optimal culture condition for Ni$^{2+}$ adsorption. During the operation course, the Cu$^{2+}$ and Ni$^{2+}$ containing wastewater flowed into stage 1 semi-continuous stirred reactor for Cu$^{2+}$ removal and recovery due to the less inhibition of Ni$^{2+}$ to Cu$^{2+}$ adsorption. The effluent of stage 1 remained large amount of Ni$^{2+}$ then flowed into stage 2 semi-continuous stirred reactor for Ni$^{2+}$ removal and recovery due to less Cu$^{2+}$ present in the effluent, hence less inhibition to Ni$^{2+}$ adsorption. The Cu$^{2+}$ and Ni$^{2+}$ bound on biosorbent in stage 1 and stage 2 was desorbed, respectively, and the regenerated biosorbent was reused, respectively, in stage 1 and stage 2. When the system reached steady state, the performances of five adsorption-desorption cycles of the series linked biosorption system was assessed. The operation results of each stage and the total treatment results for removing and recovering Cu$^{2+}$ and Ni$^{2+}$ in all cycles were listed in
Table 7.3.  

From Table 7.3, it was clearly found that in stage 1, the Cu$^{2+}$ removal efficiency reached 95% above, but the Ni$^{2+}$ was hardly removed due to the inhibition of Cu$^{2+}$ to Ni$^{2+}$ adsorption. After treatment of stage 1, the Cu$^{2+}$ concentration was reduced to 0.94 mg L$^{-1}$ to 1.34 mg L$^{-1}$ in effluent in each cycle. According to experimental results of Section 5.5, when the Cu$^{2+}$ mole concentration : Ni$^{2+}$ mole concentration was 0.1 or below in solution, Cu$^{2+}$ appeared no inhibition to Ni$^{2+}$ adsorption by *P. putida* 5-x cell. Therefore, the Ni$^{2+}$ in effluent of stage 1 could be efficiently removed in stage 2. Table 7.3 showed that Ni$^{2+}$ was obviously removed, while remained Cu$^{2+}$ in effluent of stage 1 was further reduced in biosorption of stage 2. In the final effluent, the Ni$^{2+}$ and Cu$^{2+}$ concentration were 0.91~1.09 mg L$^{-1}$ and 0.57~0.89 mg L$^{-1}$, respectively, and the total Cu$^{2+}$ and Ni$^{2+}$ removal efficiency of the two-stage biosorption system reached 97% and 96% above, respectively, in each cycle.

The Cu$^{2+}$ and Ni$^{2+}$ concentration in desorbed solution of stage 1 and stage 2 was among 0.684~0.71 g L$^{-1}$ and 0.675~0.695 g L$^{-1}$, respectively. These concentrations were high enough to be recovered by precipitation or electrolysis [Krishnan et al. 1993]. On the contrary, the Cu$^{2+}$ and Ni$^{2+}$ concentration in desorbed solution of stage 2 and stage 1 was too low to be recovered by precipitation. Preliminary analytic results indicated that above 95% of Cu$^{2+}$ and 92% of Ni$^{2+}$ in desorbed solution of stage 1 and stage 2 was recovered, respectively, by precipitation, and the content of copper and nickle in precipitate was beyond 97% and 98%, respectively. The results showed that the two-stage biosorption process with magnetite immobilized *P. putida* 5-x cell as biosorbent was efficient to sequentially remove and recover Cu$^{2+}$ and Ni$^{2+}$ from wastewater. The magnetite immobilized *P. putida* 5-x cell could be reused five cycles at least for efficiently Cu$^{2+}$ and Ni$^{2+}$ removal. The stage 1 and stage 2 biosorption mainly acted as Cu$^{2+}$ and Ni$^{2+}$
remover, respectively. In stage 1, the Cu$^{2+}$ was reduced to in trace so that stage 2 could efficiently remove Ni$^{2+}$ by adsorption due to the removal of Cu$^{2+}$ inhibition.

The major advantages of the two-stage biosorption process were: (1) the inhibition of Cu$^{2+}$ to Ni$^{2+}$ adsorption could be remove in stage 1, Cu$^{2+}$ and Ni$^{2+}$ in wastewater could be efficiently removed and recovered sequentially; (2) the Ni$^{2+}$ and Cu$^{2+}$ could be recovered individually from desorbed solution with high content without complex separation process; (3) optimal cell biomass suitable for Cu$^{2+}$ and Ni$^{2+}$ adsorption could be cultured and prepared, respectively, to provide effective Cu$^{2+}$ and Ni$^{2+}$ adsorption capacity in stage 1 and stage 2.
Table 7.3 Operation results of two series linked semi-continuous stirred reactor for sequentially removing and recovering Cu$^{2+}$ and Ni$^{2+}$ from Cu$^{2+}$ and Ni$^{2+}$ containing synthetic wastewater*

<table>
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<tr>
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</tr>
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<td>Initial Conc. (mg L$^{-1}$)</td>
<td>Cu$^{2+}$</td>
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<td>30.6</td>
<td>30.3</td>
<td>29.9</td>
</tr>
<tr>
<td></td>
<td>Ni$^{2+}$</td>
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<td>30.2</td>
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<td>Conc. in effluent of Stage 1 (mg L$^{-1}$)</td>
<td>Cu$^{2+}$</td>
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<td>Ni$^{2+}$</td>
<td>28.4</td>
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<td>29.1</td>
</tr>
<tr>
<td>Removal in Stage 1 (%)</td>
<td>Cu$^{2+}$</td>
<td>96.9</td>
<td>96.5</td>
<td>96</td>
<td>95.8</td>
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<td></td>
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<td>5.59</td>
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<tr>
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<td>Cu$^{2+}$</td>
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<td>0.696</td>
<td>0.683</td>
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<tr>
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<tr>
<td>Desorption Rate in Stage 1 (%)</td>
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<td>96.1</td>
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<td>97.1</td>
</tr>
<tr>
<td>Conc. In effluent of Stage 2 (mg L$^{-1}$)</td>
<td>Cu$^{2+}$</td>
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<td>0.71</td>
<td>0.78</td>
<td>0.83</td>
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<td>Ni$^{2+}$</td>
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<td>0.93</td>
<td>0.97</td>
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<td>96.7</td>
<td>96.8</td>
<td>96.6</td>
<td>96.4</td>
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<tr>
<td>Conc. In Desorbed Solution of Stage 2 (mg L$^{-1}$)</td>
<td>Cu$^{2+}$</td>
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<td>0.008</td>
<td>0.01</td>
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<td>Ni$^{2+}$</td>
<td>0.675</td>
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<td>Ni$^{2+}$</td>
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<td>98.5</td>
<td>99.1</td>
<td>99.2</td>
</tr>
<tr>
<td>Total Removal Passing Two Stage (%)</td>
<td>Cu$^{2+}$</td>
<td>98.1</td>
<td>97.7</td>
<td>97.4</td>
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<tr>
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<td>Ni$^{2+}$</td>
<td>96.7</td>
<td>96.9</td>
<td>96.8</td>
<td>96.6</td>
</tr>
</tbody>
</table>

* Biosorbent concentrations in stage 1 and stage 2 were 3 g L$^{-1}$. The ratio of desorption solution to biosorbent was 40 ml to 3 g biosorbent.
7.4 Sequential Removal and Recovery of Copper and Nickel from Real Electroplating Effluent Using Two Stage Biosorption Process

In order to evaluate the applicability of the two-stage biosorption process with magnetite immobilized *P. putida* 5-x cell as biosorbent for sequentially removing and recovering Cu\(^{2+}\) and Ni\(^{2+}\) from real electroplating effluent, the industrial wastewater was collected from a local electroplating plant. It averagely composed of Cu\(^{2+}\) 26.3 mg L\(^{-1}\), Ni\(^{2+}\) 22.7 mg L\(^{-1}\), SO\(_4^{2-}\) 61.2 mg L\(^{-1}\) as well as small amount of Cl\(^{-}\) (12.8 mg L\(^{-1}\)), F\(^{-}\) (2.9 mg L\(^{-1}\)), NO\(_3^{-}\) (2.6 mg L\(^{-1}\)), CN\(^{-}\) (1.9 mg L\(^{-1}\)) and Pb\(^{2+}\) (0.9 mg L\(^{-1}\)), Zn\(^{2+}\) (1.1 mg L\(^{-1}\)). Previous study on above showed that except for Zn\(^{2+}\) and Pb\(^{2+}\), other cations and anions present in wastewater did not appear significant inhibition on Cu\(^{2+}\) and Ni\(^{2+}\) adsorption. However, the concentrations of Zn\(^{2+}\) and Pb\(^{2+}\) in the electroplating effluent were too low to inhibit Cu\(^{2+}\) and Ni\(^{2+}\) adsorption. Therefore, the wastewater from electroplating effluent was directly fed into the two stage semi-continuous stirred reactor with magnetite immobilized *P. putida* 5-x cell as biosorbent for sequentially removing and recovering Cu\(^{2+}\) and Ni\(^{2+}\) without pretreatment for reducing the other ions in wastewater. According to Cu\(^{2+}\) and Ni\(^{2+}\) uptake isotherms by magnetite immobilized *P. putida* 5-x cell as below:

\[ Q_{\text{imb-Cu}} = 11.9 C_e^{0.74} \text{ and } Q_{\text{imb-Ni}} = 9.7 C_e^{0.21}. \]

if Cu\(^{2+}\) and Ni\(^{2+}\) concentrations in treated effluent are required 1 mg L\(^{-1}\) or below, the adsorption capacities of Cu\(^{2+}\) and Ni\(^{2+}\) by the immobilized cell should be 11.9 mg g\(^{-1}\) and 9.7 mg g\(^{-1}\) above. Due to Cu\(^{2+}\) and Ni\(^{2+}\) in electroplating wastewater was 26.3 mg L\(^{-1}\) and 22.7 mg L\(^{-1}\), respectively, for reducing Cu\(^{2+}\) and Ni\(^{2+}\) to 1 mg L\(^{-1}\) or below, the required biosorbent concentration in stage 1 and stage 2 should be (26.3-1.0)/11.9 = 2.12 g L\(^{-1}\) for Cu\(^{2+}\) and (22.7-1.0)/9.7 = 2.24 g L\(^{-1}\) for Ni\(^{2+}\), respectively. Allowing for potential inhibition of other cations and anions in electroplating effluent, additional biosorbent of
30% was necessary. Thus the amount of biosorbent used in stage 1 should be 2.76 g L\(^{-1}\), and in stage 2 should be 2.91 g L\(^{-1}\). For point of simplifying operation view and comparing with the operation results of synthetic wastewater, the 3 g L\(^{-1}\) of biosorbent were adopted in stage 1 and stage 2, respectively. The operation procedure of the two-stage biosorption process for treating real electroplating wastewater was similar to the procedure in Section 7.3. The treatment results of consecutive five adsorption/desorption cycles in each stage were listed in Table 7.4.

In Table 7.4, it was found generally that the treatment efficiency in each stage for Cu\(^{2+}\) and Ni\(^{2+}\) removal was low than that in synthetic wastewater. The final Cu\(^{2+}\) and Ni\(^{2+}\) removal efficiency of the two-stage biosorption process was about 96% and 94%, respectively. The lower removal efficiency comparing with synthetic wastewater might be due to a couple of reasons. With respect to the coexisting of other cations and anions, although previous study (section 5.4, and 5.6) showed that these ions alone in the concentration did not inhibited Cu\(^{2+}\) or Ni\(^{2+}\) adsorption obviously, the effect of coexisting of these ions on Cu\(^{2+}\) and Ni\(^{2+}\) adsorption might be significant. The presence of cyanide in the effluent sample, whose identity and inhibition to Cu\(^{2+}\) and Ni\(^{2+}\) adsorption have not been studied due to its toxicity, might also inhibit Cu\(^{2+}\) and Ni\(^{2+}\) uptake.

Although the removal efficiency in each stage for Cu\(^{2+}\) and Ni\(^{2+}\) was slightly low than in synthetic wastewater, the results were still acceptable. After treatment, the Cu\(^{2+}\) and Ni\(^{2+}\) concentration in final effluent was about 1.0 mg L\(^{-1}\) and 1.3 mg L\(^{-1}\), respectively. This indicated the treatment results was still acceptable, and the amount of biosorbent estimated according to uptake isotherms was proper for Cu\(^{2+}\) removal generally, but not more enough to reduce Ni\(^{2+}\) to 1 mg L\(^{-1}\) below. This might be due to the more inhibition of coexisting other ions to Ni\(^{2+}\) adsorption than to Cu\(^{2+}\) adsorption. In future application, more biosorbent are necessary for Ni\(^{2+}\) adsorption.
The Cu\textsuperscript{2+} and Ni\textsuperscript{2+} concentration in desorbed solution of stage 1 and stage 2 was among 0.564~0.59 g L\textsuperscript{-1} and 0.48~0.501 g L\textsuperscript{-1}, respectively. These concentrations were still high enough to be recovered by precipitation or electrolysis. On the contrary, the Cu\textsuperscript{2+} and Ni\textsuperscript{2+} concentration in desorbed solution of stage 2 and stage 1 was also too low to be recovered by precipitation.

These experimental data showed that only slight difference could be observed between the performance of the two-stage biosorption process with magnetite immobilized \textit{P. putida} 5-x as biosorbent for treating real industrial wastewater and synthetic Cu\textsuperscript{2+} and Ni\textsuperscript{2+} containing wastewater. Also, the final effluent of each of the five cycles could be maintained at a relatively low level of Cu\textsuperscript{2+} and Ni\textsuperscript{2+} concentration, and the concentrations of Cu\textsuperscript{2+} and Ni\textsuperscript{2+} in desorbed solution were also high enough to be recovered by precipitation. Thus the two-stage magnetite immobilized cell system is a promising technology for removing and recovering Cu\textsuperscript{2+} and Ni\textsuperscript{2+} from electropolating effluent wastewater.
Table 7.4 Operation results of two series linked semi-continuous stirred reactor for sequentially removing and recovering Cu²⁺ and Ni²⁺ from real electroplating effluent

<table>
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<th>4</th>
<th>5</th>
</tr>
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<tr>
<td>Initial Conc. (mg L⁻¹)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu²⁺</td>
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<tr>
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<tr>
<td>Cu²⁺</td>
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* Biosorvent concentrations in stage 1 and stage 2 were 3 g L⁻¹. The ratio of desorption solution to biosorvent was 40 ml to 3 g biosorbent.
Chapter 8: The Roles of Cell Surface Components of

*Pseudomonas putida 5-x on Cu$^{2+}$ Adsorption*

Experimental results in section 4.3.2 showed that the *P. putida 5-x* cell possessed high Cu$^{2+}$ and Ni$^{2+}$ adsorption capacity, but the adsorption capacities of cells in different growth ages were different due to the variation of the surface structure and components. Many studies showed that soluble metal ions in the environment were mainly captured on cell surface (cell wall or cell envelope) because of negative charged groups attached within its fabric [Doyle et al., 1980; Beveridge et al. 1982].

Gram-positive cell wall consists of a thicker peptidoglycan (PEG) layer (constitute up 40–90 % of the cell wall) into which teichoic acid (TA) and teichuronic acid (TUA) are embedded [Remacle, 1990]. PEG, TA and TUA contain large number of electronegative groups such as carboxyl and phosphodiester [Beveridge, 1986; McLean and Beveridge, 1990]. Therefore generally Gram-positive bacteria have strong interaction with cationic metal ions [Beveridge and Fyfe, 1985; Brierley, 1990]. Gram-negative bacteria have weaker metal binding capacity than Gram-positive bacteria in general due to their thinner PEG layer (only 10% of the cell envelopes) and absence of TA and TUA in cell envelopes [Brierley, 1990; Remacle, 1990]. Studies on metal binding by most Gram-negative bacteria had shown that the separated cell envelopes or outer membranes only contributed 0.001–0.4 mmol g$^{-1}$ metal binding capacities, or rather, about 10–20 % of adsorption capacity of Gram-positive bacterial cell wall [Hoyle and Beveridge, 1983; Ferris and Beveridge, 1984; Beveridge and Fyfe, 1985; Falla and Block, 1993]. On the contrary, few studies had reported that cell surface components of Gram-negative bacteria contributed a high metal binding capacity than that of Gram-positive cell wall.
Cell envelope of Gram negative bacteria consists of four major structural components: (1) capsules made of proteins and polysaccharides; (2) outer membrane made of lipopolysaccharides, proteins and lipids; (3) inner membrane made of lipids and proteins; and (4) PEG layer [Schiewer and Volesky, 2000]. The effect of capsule on Cu\textsuperscript{2+} adsorption capacity has been clarified in section 4.4.4. Since the presence of the Cu\textsuperscript{2+}-bridge in the capsule, the removal of capsule could enhance the Cu\textsuperscript{2+} adsorption capacity of cell biomass. Experimental results in section 4.4.4 had showed that 9.11g of cell without capsule was obtained from 10 g fresh cells by treating with 0.1mol L\textsuperscript{-1} HCl. Cu\textsuperscript{2+} adsorption capacity adsorption capacity increased from 67.4 mg g\textsuperscript{-1} of fresh \textit{P. putida} 5-x cells to 84.3 mg g\textsuperscript{-1} of pretreated cell, and the total Cu\textsuperscript{2+} bound has increased from 674 mg by 10 g fresh cells to 768 mg by 9.11 g pretreated cell. The adsorption isotherms of pretreated and fresh cells suggested that the pretreated \textit{P. putida} 5-x cell without capsule outside the cell surface was a better absorbent for Cu\textsuperscript{2+} removal than fresh cell.

In this chapter, Cu\textsuperscript{2+} was chosen to clarify the molecule mechanism of biosorption by \textit{P. putida} 5-x cell and the roles of other separated cell components, such as outer membrane, inner membrane and PEG layer material, on the high Cu\textsuperscript{2+} binding capacity.

8.1 Contribution of Separated Cell Components to Cu\textsuperscript{2+} adsorption

8.1.1 Cu\textsuperscript{2+} Adsorption Capacity of Separated Cell Envelope of \textit{P. putida} 5-x

Many studies on metal binding by bacteria showed that isolated walls from Gram-positive organism had higher metal binding capacity than isolated envelopes from Gram-negative bacteria, usually 5-10 times [Beveridge and Fylde, 1985; Brierley, 1990]. This is because of the difference on components and structure of cell surface between the Gram-positive and Gram-negative bacteria.
In this study, 2.41 g structure-complex stratified cell envelope materials (Figure 8.1), were obtained from 6 g fresh cell harvested in optimal culturing phase for Cu$^{2+}$ adsorption using biochemical separation technique, and the Cu$^{2+}$ adsorption capacity of the cell envelope was found to be 311 mg g$^{-1}$ in 200 mg L$^{-1}$ Cu$^{2+}$ solution at pH 6.5 containing 500 mg L$^{-1}$ biosorbent. The Cu$^{2+}$ adsorption capacity was 4 times more than that of fresh cell. The total amount of Cu$^{2+}$ bound by 2.41 g cell envelopes isolated from 6 g fresh cell was 1.4 times more than by that of 6 g fresh cells (Table 8.1). The level of Cu$^{2+}$ adsorption capacity reached up to or exceeded the level of typical Gram-positive bacterial cell wall [Beveridge and Fyfe, 1985; Brierley, 1990]. The result indicated that the cell envelope with high metal binding capacity also could be found due to the structural complexity and multiplicity of Gram-negative bacterial cell envelopes. That the total amounts of Cu$^{2+}$ bound by 2.41 g cell envelopes isolated from 6 g fresh cell exceeded by that of 6 g fresh cells indicated that partial metal-binding sites in intact cell surface were inaccessible to heavy metal ions due to the spatial obstacle. However, through chemical and physical treatments during the separation course, more metal-binding sites on cell envelopes of P. putida 5-x became accessible for effective metal binding.

Similar to the fresh cell and pretreated cell, the Cu$^{2+}$ adsorption process of isolated cell envelope also could be described with Freundlich isotherm (see Figure 8.2) as follow:

$$Q = 129C_e^{0.252}$$

K$_f$ value of the cell envelope was 4.5 times and 9.5 times more than that of pretreated cells and fresh cells, respectively. This indicated that the cell envelopes of P. putida 5-x was an excellent biosorbent for Cu$^{2+}$ removal.

In biosorption process, biosorbent regeneration, disposal and transport contribute to the major cost comparing with biosorbent production [Atkinson, 1998]. In order for
biosorption to become competitive with existing technologies. adsorption capacity of biosorbent should exceed 150 mg g\(^{-1}\) to reduce the use amount of biosorbent, and thus minimize the sludge regeneration, disposal and transport cost [Gadd, 1988]. From the adsorption capacity viewpoint, cell envelopes of \(P.\ putida\ 5\)-x is a more promising biosorbent for Cu\(^{2+}\) adsorption from industrial effluent than \(P.\ putida\ 5\)-x intact cells.
Table 8.1 Recovery rate of different cell components from fresh cells and their Cu²⁺ adsorption capacity, total Cu²⁺ binding yield *

<table>
<thead>
<tr>
<th></th>
<th>Fresh Cell</th>
<th>Cell Envelopes</th>
<th>Spheroplast Envelope</th>
<th>Outer Membrane</th>
<th>PEG Layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Dried Weight (g)</td>
<td>6</td>
<td>2.41± 0.29</td>
<td>1.85 ± 0.31</td>
<td>0.84± 0.23</td>
<td>0.236± 0.052</td>
</tr>
<tr>
<td>Content of surface components in envelope (%)</td>
<td>100</td>
<td>76.8</td>
<td>34.9</td>
<td>9.8</td>
<td></td>
</tr>
<tr>
<td>Adsorption Capacity (mg g⁻¹)</td>
<td>87.3 ± 5.2</td>
<td>311± 29</td>
<td>231 ± 17.6</td>
<td>281 ± 23</td>
<td>362 ± 22</td>
</tr>
<tr>
<td>Total Binding Yield (mg)</td>
<td>523.8</td>
<td>750</td>
<td>428</td>
<td>236</td>
<td>85.5</td>
</tr>
</tbody>
</table>

* Pretreated cells, cell envelopes and PEG layer materials were separated from 6 g fresh cells, respectively. All data is average value of three experiments. The total Cu²⁺ binding yield means total Cu²⁺ bound by total separated components derived from 6 g fresh cells.
Figure 8.1 SEM Graph of Cell Envelope of *P. putida* 5-x (x 20,000)
Figure 8.2: Adsorption Isotherms of Separated Components from Fresh Cell of
P. putida 5-x

\[ Q = 1365/1 + 3.66C_e \]

\[ Q = 129C_e^{0.233} \]

\[ Q = 109C_e^{0.233} \]

\[ Q = 99.1C_e^{0.191} \]

- ▲ Q-Env
- ■ Q-PEG
- ♦ Q-Outer
- ● Q-Plasma

Ce (ng/L)
Adorption Capacity (mg/g)
8.1.2 Cu²⁺ Adsorption by Separated Peptidoglycan Layer of P. putida 5-x Cell

In gram-positive bacteria, cell wall is majorly made up of PEG layer material, which provide the most adsorption sites for heavy metal binding. However, in gram-negative bacteria, cell envelopes consists of three major structural components: 1. outer membrane made of lipopolysaccharides, proteins and lipids; 2. inner membrane made of lipids and proteins; and 3. PEG layer [Schiewer and Volesky, 2000]. The lipids, proteins and lipopolysaccharides in outer and inner membrane can be easily dissolved by SDS solution, but the PEG layer is highly resistant to SDS extraction [Weidel and Peltzer, 1964]. From the results, around 0.238 g structure-simple mono-layer PEG layer materials, (Figure 8.3), representing 9.7 % (w/w) of cell envelopes have been separated from 6 g P. putida 5-x cell harvested in optimal culturing phase for Cu²⁺ adsorption (34h), (Table 8.1) to test its Cu²⁺ adsorption capacity and property.

Results of SDS-Page analysis (Seeing Figure 8.4), fatty acid, and diaminopimelic acid analyse showed that separated PEG layer materials only contained trace proteins and lipids, up to 95 % was of macromolecular PEGs. The adsorption capacity of PEG layer material in 200 mg L⁻¹ Cu²⁺ solution containing 500 mg L⁻¹ PEG layer materials was approximately 362 mg g⁻¹ (dry weight), which was not much more than that of whole cell envelopes. The total Cu²⁺ bound by 0.238 g PEG layer material was just 85.7 mg (Table 8.1), although the loss of PEG layer materials during the separation was inevitable, indicated that the PEG layer only contributed about 10-15 % of the Cu⁺⁺ adsorption capacity to Cu²⁺ adsorption by the cell envelope.

In addition, the Cu²⁺ adsorption of intact cell, HCl pretreated cell and cell envelopes of P. putida 5-x could be described with Freundlich isotherms. However, according to adsorption data in Figure. 8.2, the adsorption of separated PEG layer materials seemed to be better described with Langmuir isotherm as below.
\[ Q = 1365 \text{ Ce (1 - 3.60 Ce).} \]

This implied that the \( \text{Cu}^{2+} \) adsorption properties of PEG layer materials might be different from intact cells or cell envelopes.

In general, the cell walls of Gram-positive bacterial have high metal adsorption capacity due to their thicker PEG layer, in which TA and TUA are embedded. The PEG, TA and TUA all contain dense electronegative groups, such as carboxyl and phosphodiester, which bind heavy metals [McLean and Beveridge, 1990]. However, the PEG layer of the Gram-negative bacteria was much thinner (represent about 10% of the cell envelopes), and only contributed 10-15% adsorption capacity to \( \text{Cu}^{2+} \) adsorption by the cell envelope. Therefore it attracted us to find out whether other components in cell envelope of \( P.\ putida \ 5-x \), such as outer membrane and inner membrane, also contribute high \( \text{Cu}^{2+} \) binding capacity on adsorption process.
Figure 8.3  SEM Graph of Separated PEG Layer from *P. putida* 5-x Cell Envelope
(x 30,000)
Figure 8.4 Protein Analysis Results by SDS-Page

Lane 1: fresh cell, Lane 2: broken cell, Lane 3: isolated cell envelopes, Lane 4: separated PEG layer, Lane 5: Triton X-100 undissolved cell envelopes
8.1.3. Cu\(^{2+}\) Adsorption by Separated Cell Outer Membrane Material of *P. putida* 5-x

Outer membrane material was separated through treating envelope material with lysozyme and Tween-80. About 0.84 g thick outer membrane material was obtained from 6 g fresh cell of *P. putida* 5-x harvested in 34 h, Figure 8.5. The outer membrane material appeared quite high Cu\(^{2+}\) adsorption capacity of about 280 mg g\(^{-1}\) although it was still lower than PEG material. However, the outer membrane material represented 30% above of the envelope material, and the total Cu\(^{2+}\) bound by the 0.84 g outer membrane material was 236 mg, or rather, the outer membrane material contributed 31% above to Cu\(^{2+}\) adsorption by cell envelope material without considering the loss of outer membrane materials during the separation. This indicated that the total contribution of the outer membrane exceeded the PEG layer materials in Cu\(^{2+}\) adsorption by cell envelope.

Analysis results showed that the outer membrane material contained phospholipid of 37.3%, protein of 31.2% and lipopolysaccharides of 7.8%. The phospholipid content of the outer membrane material was obviously high than that in outer membrane of most gram-negative bacteria [Bobo and Eagon, 1967; Bhakoo and Herbert, 1980]. Because phospholipid provided large amounts of negative charged groups (such as phosphonate and phosphodiester), this might be the major reason of such high Cu\(^{2+}\) adsorption capacity of the outer membrane material, hence the *P. putida* 5-x cell envelopes.

Figure 8.2 showed the Cu\(^{2+}\) adsorption of the outer membrane materials also obeyed the *Freundlich* isotherm as below:

\[
Q = 109 C_e^{0.253}
\]

The K value of 109 indicated that the outer membrane was also a better biosorbent for Cu\(^{2+}\).
Figure 8.5 SEM Graph of Separated Cell Outer Membrane of *P. putida* 5-x (x 20,000)
8.1.4. Contribution of Inner Membrane Material on Cu$^{2+}$ Adsorption

The separated inner membrane material was not obtained due to the difficulty of separation course. but the spheroplast envelope material was obtained through treating envelope with lysozyme. The spheroplast envelope material is made up of outer and inner membrane. If the Cu$^{2+}$ adsorption capacity, weight of spheroplast envelope and outer membrane were determined, the Cu$^{2+}$ adsorption capacity, weight of inner membrane material could be calculated as follows:

\[ W_{\text{inner}} = W_{\text{spheroplast}} - W_{\text{outer}} \]  
\[ Q_{\text{inner}} = (Q_{\text{spheroplast}} W_{\text{spheroplast}} - Q_{\text{outer}} W_{\text{outer}}) W_{\text{inner}} \]

where \( W_{\text{inner}} \), \( W_{\text{spheroplast}} \) and \( W_{\text{outer}} \) are weight of inner membrane, spheroplast envelope and outer membrane in envelope. (g); \( Q_{\text{inner}} \), \( Q_{\text{spheroplast}} \) and \( Q_{\text{outer}} \) are the Cu$^{2+}$ adsorption capacity of inner membrane, spheroplast envelope and outer membrane (mg g$^{-1}$).

In addition, if the content of proteins and phospholipids in outer membrane and spheroplast envelope, the content of proteins and phospholipids in inner membrane can be estimated by following equations:

\[ C_{\text{pro-in}} = (C_{\text{pro-plasma}} W_{\text{spheroplast}} - C_{\text{pro-outer}} W_{\text{outer}}) W_{\text{inner}} \]  
\[ C_{\text{P-lipd-in}} = (C_{\text{P-lipd-spheroplast}} W_{\text{spheroplast}} - C_{\text{P-lipd-outer}} W_{\text{outer}}) W_{\text{inner}} \]

where \( C_{\text{pro-in}} \), \( C_{\text{pro-spheroplast}} \) and \( C_{\text{pro-outer}} \) are the content of proteins in inner membrane, spheroplast envelope and outer membrane (°); \( C_{\text{P-lipd-in}} \), \( C_{\text{P-lipd-spheroplast}} \) and \( C_{\text{P-lipd-outer}} \) are the phospholipid content in inner membrane, spheroplast envelope and outer membrane (°).

Certainly, these calculated data was just a theoretic value, the error with real value was inevitable. Although that, the studies based to these calculated data could serve as an
useful aid for preliminarily clarifying the role and characteristic of inner membrane in 
\( \text{Cu}^{2+} \) adsorption by \( P. \text{putida} \) 5-x cell envelope.

About 1.85 g spheroplast envelope material was obtained from 6 g fresh cell of \( P. \text{putida} \) 5-x 
harvested in 34 hr. From Figure 8.6, it was clearly found that spheroplast envelope was 
composed of two thick membrane layers. An interspace was observed between the two 
membrane layers, this was because the PEG layer between the outer membrane and inner 
membrane was degraded by lysozyme. Experimental results showed that the \( \text{Cu}^{2+} \) adsorption 
capacity of the plasma membrane material was about 230 mg g\(^{-1}\). The adsorption of the 
spheroplast envelope was also obeyed the \textit{Freundlich} isotherm (seeing Figure 8.2) as below

\[
Q = 99.1C_{e}^{0.191}
\]

According to weight and \( \text{Cu}^{2+} \) adsorption capacity of spheroplast envelope and outer 
membrane, the weight and \( \text{Cu}^{2+} \) adsorption capacity of the inner membrane was determined to 
be 1.01 g and 188 mg g\(^{-1}\), respectively according to equation I and II. This result appeared that 
the \( \text{Cu}^{2+} \) adsorption capacity of inner membrane was lower than that of outer membrane, while 
the content of inner membrane in cell envelope was more than outer membrane. The total \( \text{Cu}^{2+} \) 
bound by the 1.01g inner membrane material was about 190 mg, or rather, the inner membrane 
material contributed 25 % above to \( \text{Cu}^{2+} \) adsorption by cell envelope material. The total 
contribution of the inner membrane was low to outer membrane and high to the PEG layer 
in \( \text{Cu}^{2+} \) adsorption by cell envelope.

According to analysis results of phospholipid and protein in spheroplast envelope and outer 
membrane, the content of phospholipid and protein in inner membrane was determined, that 
was 16.7 % and 45.5 %, respectively. Clearly, the phospholipid content in inner membrane was 
lower than in outer membrane, while protein content were higher than in outer membrane. This 
result indicated, comparing with \( \text{Cu}^{2+} \) adsorption capacity and ingredients content of outer
membrane, lower phospholipid content and absence of lipopolysaccharides might result in the lower Cu$^{2+}$ adsorption capacity of inner membrane. The variation of proteins content between inner and outer membrane seemed to be irrespective to Cu$^{2+}$ adsorption capacity of inner membrane or outer membrane materials. Although the content of lipopolysaccharides was relatively low than phospholipid in outer membrane, the dense carbonyl and carboxyl groups attached on lipopolysaccharides could contribute more negative charged groups for heavy metal binding [Rogers et al. 1968; Wilkinson et al. 1973].
Figure 8.6 SEM Graph of Cell Spheroplast Envelope of *P. putida* 5-x (x 30,000)
The experimental results showed that cell envelopes of *P. putida* 5-x possessed high Cu$^{2+}$ adsorption capacity, which reached up or exceeded the adsorption capacity of general Gram-positive cell wall [Beveridge and Fyfe, 1985; Brierley, 1990]. All of the PEG layer, outer membrane and inner membrane contributed to Cu$^{2+}$ adsorption capacity in Cu$^{2+}$ adsorption by the cell envelopes. In the Cu$^{2+}$ adsorption of cell envelope separated from 34 hr-fresh cell, the Cu$^{2+}$ adsorption capacity of PEG layer, outer membrane and inner membrane was in order of PEG > Outer membrane > Inner membrane, while the component content in envelope was in order of inner membrane > outer membrane > PEG layer. Considering both content and Cu$^{2+}$ adsorption capacity of these components in cell envelope, the contributions of these components to Cu$^{2+}$ adsorption was in order of outer membrane > inner membrane > PEG layer.

According to these results, we think if the content of PEG layer and outer membrane in cell envelope could be increased, the adsorption capacity of cell envelope would be enhanced obviously.
8.2: Effect of Variation of Cell Surface Components along with Cell Growth Age on Cu^{2+} Adsorption Capability of P. putida 5-x Cell Envelope

Results of Section 4.3.2 indicated that the Cu^{2+} adsorption capacity of the P. putida 5-x cell in different growth phases was different due to the variation of cell surface structure and components. The results in Section 8.1 showed that all cell surface components such as PEG layer, outer membrane and inner membrane, played important part in the adsorption of the cell envelopes. This meant that the variation of Cu^{2+} adsorption capacity of P. putida 5-x cell or its cell envelopes along with cell growth age mainly depended on the variation of these cell components.

The cell envelope of gram-negative bacteria consists of three major structural components: (1) outer membrane made of lipopolysaccharides, proteins and lipids; (2) inner membrane made of lipids and proteins; and (3). Peptidoglycan (PEG) layer [Schiewer and Volesky, 2000]. Thus the total Cu^{2+} bound by separated cell envelope of P. putida 5-x could be expressed by equation (V), and the adsorption capacity of the cell envelope could be expressed as equation (VI).

\[ W_{envelope} \cdot Q_{envelope} = W_{outer} \cdot Q_{outer} + W_{inner} \cdot Q_{inner} - W_{PEG} \cdot Q_{PEG} \]  
\[ Q_{envelope} = \left( Q_{outer} \cdot W_{outer} + Q_{inner} \cdot W_{inner} - Q_{PEG} \cdot W_{PEG} \right) \cdot W_{envelope} \]

where, \( W_{envelope}, W_{outer}, W_{inner} \) and \( W_{PEG} \) are the dry weight of cell envelope, outer membrane, inner membrane, and PEG layer isolated from fresh cell, and \( Q_{envelope}, Q_{outer}, Q_{inner} \) and \( Q_{PEG} \) are the adsorption capacities of cell envelope, outer membrane, inner membrane, and PEG layer in different cell growth phases. Certainly, the equations just are the theoretic formulas. The errors between the calculated data according to these theoretic formulas and real value are inevitable. Although that, the formulas can serve as
a useful aid to simplify the problems we studied.

For clarifying the mechanism of the variation of Cu$^{2+}$ adsorption capacity along with cell growth age, which can serve as useful aids for modifying cell surface component using metabolic regulation and DNA recombination technology to provide much better biosorbent for Cu$^{2+}$, cell envelope and various cell surface components were separated from fresh *P. putida* 5-x cell with different growth ages, and their content and Cu$^{2+}$ adsorption capacities were determined, respectively. The effect of variation of content and adsorption capacity of cell components in different cell growth phases on Cu$^{2+}$ adsorption capacity of cell envelope was assessed. In addition, the content of major ingredients in the outer membrane and inner membrane of cells in different growth ages, such as phospholipid, protein, lipopolysaccharides, were analyzed and the effect of their variation on Cu$^{2+}$ adsorption capacity of outer and inner membrane was studied.

### 8.2.1 Variation of Content of Cell Surface Components and Their Cu$^{2+}$ Adsorption Capacities in Different Cell Growth Phases

Cell envelopes and cell surface components, such as outer membrane, spheroplast envelope and PEG layer were separated from 6 g fresh cell, respectively, harvested in different cell ages. The adsorption capacity of cell envelope and the content of outer membrane, spheroplast envelope and PEG layer in cell envelope in different cell growth phases was determined, (the content of inner membrane and its adsorption capacity were estimated by equation I and II). The results were shown in Figure 8.7. It was found that the adsorption capacity of cell envelope and the content of PEG layer, outer and inner membrane in cell envelope in different growth phases were different. After culturing 12 hr, the adsorption capacity of cell envelope decreased gradually, and the lowest point of
adsorption capacity appeared at about 20 hr. But after 20 hr, the adsorption capacity of cell envelope began to increase again, and accelerated rise after 30 hr, the highest point appeared at about 36 hr, then decreased again. Regarding outer membrane content, before culturing 18 hr, the content increased gradually, but after 18 hr, began to decrease gradually along with cell growth. The top content of outer membrane appeared at 18 hr with representing 56.8 % of cell envelope. For inner membrane, the content was generally constant, but when cell growth entered stationary phase, (after 24 hr), the content had an obvious increment. The peak of content appeared at 36-38 hr (end of stationary phase) with representing 41.9 % of cell envelope. For PEG layer, the variation tendency of content along with cell growth was similar to the variation tendency of adsorption capacity of cell envelope, both lowest points and top points appeared at around 20 hr and 36 hr, respectively, (Figure 8.7).

Above experimental results showed that the content of each cell surface component in different cell growth phases was obviously various. From equation VI, it was known that the variation of the content of cell components along with cell growth age consequentially resulted in the difference of Cu^{2+} adsorption capability of P. putida 5-x cell envelope in different cell growth phases. The results in Table 8.1 indicated that the adsorption capacities of PEG layer, outer membrane and inner membrane isolated from fresh cell was in order of PEG layer (386 mg g^{-1}) > outer membrane (307 mg g^{-1}) > inner membrane (217 mg g^{-1}). If the Cu^{2+} adsorption capacity of each cell component isolated from P. putida 5-x cell in different cell ages was constant, the variation of adsorption capacity of cell envelope should only depend on the variation of content of PEG layer, outer membrane and inner membrane. Thus the cell envelope isolated from the cell growth phase which contain a large amount of PEG layer and outer membrane in cell envelope should have the highest adsorption capacity to Cu^{2+}. However, it was found
from Figure 8.7 that the real highest and lowest point of adsorption capacity of cell envelope appeared at about 18 hr and 36 hr, respectively, but the calculated highest and lowest point of adsorption capacity according to equation VI appeared at 12 hr and 18 hr, supposing the adsorption capacities of PEG layer, outer membrane and inner membrane in different growth phases constantly were about 386 mg g⁻¹, 307 mg g⁻¹ and 217 mg g⁻¹, respectively. The difference of variation tendencies between the real adsorption capacity of cell envelope and the calculated adsorption capacity of cell envelope according to equation VI along with cell growth age implied that the adsorption capacity of PEG layer, outer membrane and inner membrane might be not constant in different cell growth phases. Thus, the adsorption capacities of separated PEG layer, outer membrane and inner membrane from fresh cell harvested in different cell growth phases were studied as below.

The Cu²⁺ adsorption capacity of separated outer membrane, spheroplast envelope and PEG layer isolated from fresh cell with different cell ages was tested, respectively, and the adsorption capacity of inner membrane was estimated by equation II. The results in Figure 8.8 clearly showed that the Cu²⁺ adsorption capacities of outer membrane and inner membrane in different growth phases are various obviously, while the adsorption capacity of PEG layer is constant generally in different growth phases. According to equation VI, the adsorption capacity of cell envelope subjects to the content of PEG layer, outer and inner membrane in cell envelope and their adsorption capacities. However, the experiment showed that the content of PEG layer, outer and inner membrane, and the adsorption capacity of outer and inner membrane in different growth phases was various, while the adsorption capacities of PEG layer was constant. Thus, the variation of adsorption capacity of cell envelope in different growth phases subjects to the variation of content of PEG layer, outer and inner membrane, and variation of
adsorption capacities of outer and inner membrane along with cell age, but did not related to adsorption capacity of PEG layer. In addition, it could be found in Figure 8.7 and Figure 8.8 that the variation tendency of $\text{Cu}^{2+}$ adsorption capacity of cell envelope along with cell growth age was obviously in step with variation tendencies of adsorption capacity of outer membrane and content of PEG layer along with cell age. This implied that the variation of adsorption capacity of outer membrane and the content of PEG layer along with cell age played a major role on variation of adsorption capacity of cell envelope along with growth phase.

From the content and adsorption capacities of PEG layer, outer and inner membrane in different cell growth phases, the calculated adsorption capacities of cell envelope in different growth phases were obtained according to equation VI. It was found in Figure 8.8 that although the calculated adsorption capacity of cell envelope was low than real cell envelope in each growth phase, the variation tendency of calculated adsorption capacity of cell envelope was obviously in step with that of real cell envelope. This further confirmed that the variation of adsorption capacity of cell envelope not only depends on the variation of content of PEG layer, outer and inner membrane in cell envelope, but also on the variation of adsorption capacities of outer and inner membrane along with cell growth age. The difference between calculated adsorption capacity and real adsorption capacity of cell envelope might be due to the recovery rate of PEG layer, outer and inner membrane during the separation process did not reach 100 %, particular in late growth phase.

According to above results we think that the phase which contains a large amount of PEG layer and outer membrane, while the adsorption capacity of outer and inner membrane is at relatively high level is an optimal harvest phase during the $P.\ putida$ 5-x cell culturing as biosorbent for $\text{Cu}^{2+}$.
PEG layer is composed of single material, that is PEG, thus, the structure and property of PEG layer in different growth phases is constant, hence its adsorption capacity to Cu$^{2+}$ is generally constant in different growth phases. However, the outer and inner membrane was made up of many materials such as proteins, phospholipids, lipopolysaccharides. In different cell culture conditions, the ratio of proteins: phospholipids: lipopolysaccharides in outer or inner membrane is different. Thus the variation of adsorption capacity of outer and inner membrane along with cell growth phase certainly related to the variation of ratio of proteins: phospholipids: lipopolysaccharides in outer and inner membrane along with cell growth phase. In next section, the effect of variation of content of proteins, phospholipids and lipopolysaccharides in outer and inner membrane along with cell growth age on their Cu$^{2+}$ adsorption capacity was studied.
Fig 8.7. Adsorption capacity of cell envelope and content of cell surface components in different cell growth phases

- Outer membrane (%)
- Inner Membrane (%)
- PEG (%)
- Envelope-Ac (mg g⁻¹)
- Cal-Env-Ac/Cons (mg g⁻¹)

Envelope-Ac --- Real adsorption capacity of cell envelope in different growth phases (mg g⁻¹)

Cal-Env-Ac/Cons --- Calculated adsorption capacity of cell envelope in different growth phases according to equation II, supposing the adsorption capacities of PEG layer, outer and inner membrane are constant in different growth phases.
Figure 8.8: Copper Adsorption Capacity of Cell Components Separated from Cell Harvested in Different Growth Phases

Envelope-Ac --- Real adsorption capacity of cell envelope in different growth phases (mg g⁻¹)

Envelope-Ac (Calu) --- Calculated adsorption capacity of cell envelope in different growth phases according to equation II.
8.2.2 Effect of Variation of Proteins, Phospholipids and Lipopolysaccharides Content in Outer and Inner Membrane on Their Cu\textsuperscript{2+} Adsorption Capacity

Experimental results in section 8.2.1 showed that Cu\textsuperscript{2+} adsorption capacities of outer and inner membrane in different cell growth ages were different. Outer membrane and inner membrane are made up of proteins, phospholipids, lipopolysaccharides, and proteins, phospholipids, respectively. Thus, certainly the variation of Cu\textsuperscript{2+} adsorption capacity of outer and inner membranes in different cell growth ages related to these activity ingredients content in outer and inner membrane. In this section, the content of proteins, phospholipids and lipopolysaccharides in outer membrane and spheroplast envelope in different cell growth ages were analyzed, and the content of protein and phospholipid in inner membrane were estimated by equation III and IV. The relationships between these ingredients content and Cu\textsuperscript{2+} adsorption capacity of outer and inner membranes were studied in different cell growth phases.

Figure 8.9 showed the Cu\textsuperscript{2+} adsorption capacity of outer membrane, and proteins content, phospholipids content and lipopolysaccharides content in outer membrane in different cell growth phases. It was clear that these ingredients content in outer membrane were obvious various in different cell growth phases. The variation tendencies of phospholipids content and lipopolysaccharides content in different growth phases were similar to the variation tendency of Cu\textsuperscript{2+} adsorption capacity of outer membrane, while the variation tendency of proteins content in different growth phases was contrary to the variation tendency of Cu\textsuperscript{2+} adsorption capacity of outer membrane. This result clearly indicated that the variation of Cu\textsuperscript{2+} adsorption capacity of outer membrane along with cell growth age mainly depended on the variation of phospholipids content and lipopolysaccharides content in outer membrane, proteins seemed to play a minor role on Cu\textsuperscript{2+} adsorption of outer membrane. Corresponding to lowest point at 16-18 hr and
highest point at 36 hr of phospholipids content and lipopolysaccharides content, outer membrane appeared minimum Cu\(^{2+}\) adsorption capacity of 149 mg g\(^{-1}\) and maximum Cu\(^{2+}\) adsorption capacity of 275 mg g\(^{-1}\), respectively.

It was known that in lower pH, lipopolysaccharides and phospholipids contain large amount of negative charged groups, such as carbonyl, carboxyl, phosphonate and phosphodiester, which could effectively bind positive charged metal ions in wastewater at lower pH. Thus, the more the lipopolysaccharides and phospholipids in outer membrane, the high the Cu\(^{2+}\) adsorption capacity. Regarding to proteins, although contains some active groups heavy metal binding related, such as hydroxyl, sulphydryl, amine and amide, they are positive charged in lower pH (pH<8.0). Only when pH >8~9, they appear negative charged to contribute cations adsorption capability. Therefore, in the lower pH solution the Cu\(^{2+}\) adsorption capacity of P. putida 5-x cell was lower than in the higher pH solution because of proteins not providing negative charged groups for heavy metal adsorption.

For inner membrane, it was clearly found in Figure 8.10 that both Cu\(^{2+}\) adsorption capacity and phospholipids content of inner membrane decreased gradually, while proteins content increased along with cell growth age. This result illustrated that the phospholipids played a major role in Cu\(^{2+}\) adsorption of inner membrane and proteins appeared a minor role. The variation of Cu\(^{2+}\) adsorption capacity of inner membrane in different cell growth phase was mainly depended on the variation of phospholipids content in inner membrane.

According to experimental results in this chapter, it could be concluded that PEG layer, outer membrane and inner membrane all played important part in Cu\(^{2+}\) adsorption by cell envelopes of P. putida 5-x. The phospholipids and lipopolyusaccharides provided main negative charged groups for heavy metal adsorption of outer membrane, while
phospholipids and PEG provided main metal binding sites for heavy metal adsorption by inner membrane and PEG layer materials, respectively. Proteins in both outer membrane and inner membrane only played a minor role for heavy metal binding.

Above experimental results indicated that if content of phospholipid and lipopolysaccharides in outer membrane, and phospholipid in inner membrane could be increased using metabolism regulation technique or DNA recombination technique, the adsorption capacity of outer and inner membrane would be enhanced, hence, the adsorption capacity of cell envelope could be enhanced.
Figure 8.9 Adsorption capacity and ingredient content of outer membrane in different cell growth phases

* Outer Memb.-AC —— Cu$^{2+}$ adsorption capacity of outer membrane (mg g$^{-1}$)
Figure 8.10: Adsorption Capacity and Ingredient Content of Inner Membrane in Different Cell Growth Phases

* Inner Memb.-AC ------ $\text{Cu}^{2+}$ adsorption capacity of inner membrane (mg g$^{-1}$)
Chapter 9: General Discussion

9.1 Selection and Preparation of Biosorbent, Development of Biosorption Reactor and Study of Biosorption Mechanism

As mentioned in introduction, biosorbent regeneration, transport and disposal contributed to major cost to biosorption compared with biosorbent production and operation [Atkinson et al., 1998]. In order for biosorption to become competitive with existing technology, metal adsorption capacity of biomass should be improved as far as possible to reduce the using amount of biosorbent and thus minimizing the sludge regeneration, disposal and transport cost. For improving adsorption capacity of biosorben for heavy metal, the screening of bacteria with high Cu\(^{2+}\) and Ni\(^{2+}\) adsorption capacity, the optimization of biosorbent preparation techniques, and the optimal adsorption conditions and process design were studied in this project.

In addition, for providing a useful aid for further enhancing metal adsorption capacity of bacterial cells through modifying cell surface structure and components using either DNA recombination technology or metabolic regulation techniques, the biosorption mechanisms, such as adsorption site and active groups adsorption related, and the contribution of cell surface components and chemical ingredients to heavy metal adsorption, and the adsorption isotherms, were also studied.

In this project a bacterial strain *P. putida* 5-x which is of high adsorption capacity to both Cu\(^{2+}\) and Ni\(^{2+}\) was isolated from electroplating effluent as biosorbent using novel plate screening method. The novel plate screening method was developed and modified by us based on the Thomas’s methods (1990). The screening results indicated the modified screening method was efficient to select bacterial cell with high adsorption capacity of
heavy metal. The Cu\(^{2+}\) and Ni\(^{2+}\) adsorption capacity of the isolated P. putida 5-x cell was further enhanced through optimization of cell preparation conditions and application of cell pretreatment techniques. Factors affecting biosorption of cell biomass have been systematically and extensively studied, namely, the effects of cell culture condition cell age, biomass pretreatment, solution pH, competing cations and anions and adsorption temperature on adsorption. Experimental results indicated, although the biosorbent has relatively high adsorption capacity to heavy metal than conventional adsorbent, in its optimal adsorption conditions, about 30-40 % of adsorption capacity to Cu\(^{2+}\) and Ni\(^{2+}\) also could be increased.

For exhibiting of adsorption capability of P. putida 5-x to Cu\(^{2+}\) and Ni\(^{2+}\), a novel and economical two stage semi-continuous biosorption reactor was developed in this project for cooperating with P. putida 5-x to remove and recover Cu\(^{2+}\) and Ni\(^{2+}\) from electroplating effluent. Experimental results showed, cooperating with this biosorption reactor, the P. putida 5-x cell as biosorbent could be reused five cycles at least to efficiently remove and recover Cu\(^{2+}\) and Ni\(^{2+}\) sequentially from electroplating effluent. This indicated the semi-continuous biosorption reactor was quite efficient for P. putida 5-x cell as biosorbent for removing and recovering Cu\(^{2+}\) and Ni\(^{2+}\). The major advantages of the two-stage biosorption process were: (1) the adsorption capability of P. putida 5-x cell could be efficiently exhibited; (2) the inhibition of Cu\(^{2+}\) to Ni\(^{2+}\) adsorption could be remove in stage 1, and the Cu\(^{2+}\) and Ni\(^{2+}\) in wastewater could be efficiently removed and recovered sequentially; (3) the Ni\(^{2+}\) and Cu\(^{2+}\) could be recovered individually from desorbed solution with high content without complex separation process; (4) optimal cell biomass suitable for Cu\(^{2+}\) and Ni\(^{2+}\) adsorption could be cultured and prepared, respectively, to provide effective Cu\(^{2+}\) and Ni\(^{2+}\) adsorption capacity in stage 1 and stage 2.

Clear understanding of the biosorption mechanisms, such as adsorption sites and active
groups on the cell surface, contribution of cell surface components and chemical
ingredients to heavy metal adsorption, biosorption kinetics, the uptake capacity and
affinity of bacterial cell biomass to Cu\(^{2+}\) and Ni\(^{2+}\), and the adsorption isotherm is very
crucial for developing, improving and controlling microbial adsorption processes. Thus,
an extensive study on the biosorption mechanisms was also investigated in this project.

The role of cell surface components on Cu\(^{2+}\) adsorption by cell envelope was studied in
detail, this is a key innovative elements in this thesis. Because few studies on this scope
had been reported previously in detail. Clarifying the contributions of cell surface
components, such as PEG layer, outer and inner membrane, to Cu\(^{2+}\) adsorption of cell
envelope could provide a useful aid for modifying and optimizing cell surface component
and structure through DNA recombination technology or metabolic regulation to further
enhance the adsorption capacity of cell envelope.

The experimental results showed that cell envelopes of \(P.\ putida\ 5-x\) possessed high Cu\(^{2+}\)
adsorption capacity, which reached up or exceeded the adsorption capacity of general
Gram-positive cell wall [Beveridge and Fyfe, 1985; Brierley, 1990]. All of the PEG layer,
outer membrane and inner membrane contributed to Cu\(^{2+}\) adsorption capacity in Cu\(^{2+}\)
adsorption by the cell envelopes. This might be the main reason of such high adsorption
capacity of cell envelope of \(P.\ putida\ 5-x\). However, the Cu\(^{2+}\) adsorption capacity of PEG
layer, outer membrane and inner membrane was in order of PEG> Outer membrane>
Inner membrane, while the cell components content in envelope was in order of inner
membrane > outer membrane > PEG layer. Considering both content and Cu\(^{2+}\) adsorption
capacity of these components in cell envelope, the contributions of these components to
Cu\(^{2+}\) adsorption was in order of outer membrane > inner membrane > PEG layer.

According to these results, we think that if the content of PEG layer and outer membrane
in cell envelope could be increased through DNA recombination or metabolic regulation
technology, the adsorption capacity of cell envelope would be enhanced obviously.

In addition, Experiments found that Cu\(^{2+}\) adsorption capacity of the outer membrane was much higher than those in previous reports, they generally were 0.01-0.4 mmol g\(^{-1}\) [Hoyle and Beveridge, 1983; Remacle, 1990], this might be the main reason of such high adsorption capacity of cell envelope of P. putida 5-x. Analysis results showed that the outer membrane material contained phospholipids of about 39.3 %, proteins of 31.2 % and lipopolysaccharides of 7.8 %. The phospholipids content in the outer membrane material was relatively higher compared with the outer membrane of most of Gram-negative bacteria [Bobo and Eagon, 1967; Bhakoo and Herbert, 1980]. Because the phospholipid provide large amounts of negative charged groups (such as phosphonate and phosphodiester), the outer membrane containing high content of phospholipid have high Cu\(^{2+}\) adsorption capacity, hence the adsorption capacity of the cell envelope is high. Although the content of lipopolysaccharides was relatively low than phospholipids in the outer membrane, the dense carbonyl and carboxyl groups attached on lipopolysaccharides could also contribute more negative charged groups for heavy metal binding.

The phospholipids content in inner membrane was lower than that in outer membrane, while proteins content was higher than that in outer membrane. This result implied that, comparing with outer membrane, lower phospholipids content and absence of lipopolysaccharides might result in a lower Cu\(^{2+}\) adsorption capacity of inner membrane.

The experimental results showed that the phospholipids and lipopolysaccharides provided main negative charged groups for heavy metal adsorption of outer membrane, while phospholipids and PEG provided main negative charged groups for heavy metal adsorption by inner membrane and PEG layer materials, respectively. Proteins in both outer membrane and inner membrane only played a minor role for heavy metal binding. It was known that even in lower pH solution, lipopolysaccharides and phospholipids also
contain large amount of negative charged groups, such as carbonyl, carboxyl, phosphonate and phosphodiester [Schiewer and Volesky, 2000], which could effectively bind positive charged metal ions in wastewater at lower pH. Thus, the more the lipopolysaccharides and phospholipids in outer membrane, the high the Cu\(^{2+}\) adsorption capacity of outer membrane. Regarding to proteins, although it contains some active groups heavy metal binding related, such as hydroxyl, sulfhydryl, amine and amide, they are positive charged in lower pH (pH<8.0). Only when pH>8~9, they appear negative charged to contribute to cations adsorption [Schiewer and Volesky, 2000]. Therefore, generally, in low pH solution, the Cu\(^{2+}\) adsorption capacity of *P. putida* 5-x cell was lower than in higher pH solution. In this study, the Cu\(^{2+}\) adsorption process was carried out in pH 6-6.5 solution, thus the proteins did not play an important role.

According to above discussions, we think if content of phospholipid and lipopolysaccharides in outer membrane, and phospholipid in inner membrane could be increased using metabolism regulation technique or DNA recombination technique, the adsorption capacity of outer and inner membrane would be enhanced, hence, the adsorption capacity of cell envelope could be enhanced.

**9.2 The Main Innovative Elements and Originality of the Research Work**

In this project, a novel biosorbent - *P. putida* 5-x cell and novel biosorption process was developed to remove and recover the Cu\(^{2+}\) and Ni\(^{2+}\) from electroplating effluent by the means of biosorption. In addition, the mechanism of biosorption by *P. putida* 5-x cell and the adsorption site on cell surface were studied in detail. Although the biosorption technology is not originally new, the research work also contained some innovative elements and original work as follows:
(1): A rapid plate screening method was modified and developed based on the Thomas's methods (1994) to efficiently select the bacteria with high adsorption capacity of Cu$^{2+}$ and Ni$^{2+}$ from electroplating effluent. Experimental results indicated that the almost of strains selected by this screening method have high adsorption capacity of Cu$^{2+}$ and Ni$^{2+}$. This illustrated that the modified screening method was efficient and applicable in selection of biosorbent.

(2): Previously, many reports had indicated that gram-positive bacteria have much higher adsorption capacity of heavy metal than gram-negative bacteria due to gram-positive bacteria have thicker PEG layer in their cell wall. However, in this project a gram-negative bacterium- *P. putida* 5-x cell with much high adsorption capacity of Cu$^{2+}$ and Ni$^{2+}$ was selected using rapid plate screening method. The adsorption capacity of the *P. putida* 5-x cell to Cu$^{2+}$ and Ni$^{2+}$ reached up or exceeded the adsorption capacity of typical gram-positive bacteria.

(3): A novel two stage semi-continuous biosorption reactor was developed to sequentially remove and recover the Cu$^{2+}$ and Ni$^{2+}$ from electroplating effluent. The novel two stage semi-continuous biosorption reactor have some advantages compared with continuous column reactor or batch stirred reactor as follows:

(i) the adsorption capability of *P. putida* 5-x cell could be efficiently exhibited in this novel bioreactor; (ii) the inhibition of Cu$^{2+}$ to Ni$^{2+}$ adsorption could be remove in stage 1, Cu$^{2+}$ and Ni$^{2+}$ in wastewater could be efficiently removed and recovered sequentially; (iii) the Ni$^{2+}$ and Cu$^{2+}$ could be recovered individually from desorbed solution with high content without complex separation process; (iv) optimal cell biomass suitable for Cu$^{2+}$ and Ni$^{2+}$ adsorption could be cultured and prepared, respectively, to provide effective Cu$^{2+}$ and Ni$^{2+}$ adsorption capacity in stage 1 and stage 2.
(4): the contribution of cell surface components of \textit{P. putida} 5-x, such as capsule, PEG layer, outer and inner membrane, to \(\text{Cu}^{2+}\) adsorption of cell envelope was first studied in this project. This is a key innovative element in the thesis, because few studies on this scope had been reported previously in detail. Experimental results indicated that the presence of capsule on cell surface inhibited the interaction between \(\text{Cu}^{2+}\) and binding site on outer membrane and PEG layer, thus fresh cell has lower adsorption capacity of \(\text{Cu}^{2+}\) than pretreated cell. The PEG layer, outer and inner membrane all contributed to \(\text{Cu}^{2+}\) adsorption by cell envelope, their adsorption capacity to \(\text{Cu}^{2+}\) was in order of PEG layer > outer membrane > inner membrane, but their content in cell envelope was in order of Inner membrane > outer membrane > PEG layer. According to these results, we think if the content of PEG layer and outer membrane in cell envelope could be increased through DNA recombination technology or metabolism regulation, the adsorption capacity of cell envelope would be enhanced obviously.

(5): the roles of function materials such as proteins, phospholipid and lipopolysaccharides in outer and inner membrane on \(\text{Cu}^{2+}\) adsorption by outer or inner membrane were studied. This is also an innovative element in this project. Experimental results indicated the phospholipid and lipopolysaccharides played a major part on \(\text{Cu}^{2+}\) adsorption by outer membrane, while phospholipid played a major part on \(\text{Cu}^{2+}\) adsorption by inner membrane. Proteins seemed to play a minor part on \(\text{Cu}^{2+}\) adsorption by outer or inner membrane. According to this result, we think if phospholipid and lipopolysaccharides content in outer membrane, or phospholipid content in inner membrane could be increased during the cell culture period, the adsorption capacity of outer and inner membrane would be enhanced obviously, hence the adsorption capacity of cell envelope would also be enhanced.

9.3 Main Reasons of Such High \(\text{Cu}^{2+}\) Adsorption Capacity of Gram-Negative
Bacterium *P. putida* 5-x Cell

In previous, many studies reported that gram-negative bacteria have weaker adsorption capability than positive bacteria due to their thin PEG layer and absence of TA and TUA. However, in this project, a gram-negative bacterium *P. putida* 5-x with high Cu$^{2+}$ and Ni$^{2+}$ accumulating capability was isolated from electroplating effluent. The adsorption sites and adsorption mechanism of *P. putida* 5-x cell was widely studied, and the main reasons of such high Cu$^{2+}$ adsorption capacity of the *P. putida* 5-x cell was also studied and discussed in this project.

Experimental results indicated that all cell surface components, such as PEG layer, outer membrane and inner membrane contributed to Cu$^{2+}$ adsorption of *P. putida* 5-x. The content of PEG layer, outer membrane and inner membrane in cell envelope was in order of inner membrane > outer membrane > PEG layer, and their Cu$^{2+}$ adsorption capacities was in order of PEG layer > outer membrane > inner membrane. Both considering the content of cell surface components in envelope and their Cu$^{2+}$ adsorption capacity, the total contribution of PEG layer, outer membrane and inner membrane to Cu$^{2+}$ adsorption by cell envelope was in order of outer membrane > inner membrane > PEG layer. Compared with outer membrane in the most of gram-negative bacteria, the adsorption capacity of the outer membrane was obviously high. Thus the adsorption capacity of the cell envelope was much high than that in the most of gram-negative bacteria. Experimental results indicated that phospholipids and lipopolysaccharides contributed main adsorption groups to Cu$^{2+}$ adsorption, and proteins only played a minor part on Cu$^{2+}$ adsorption by outer membrane. Analytic results indicated that the content of phospholipids in the outer membrane was obviously higher than that in most of outer membrane of gram-negative bacteria, and the lipopolysaccharides content was slightly higher than that in the most of outer membrane of gram-negative bacteria. This might be the main reason of such high adsorption.
capacity of outer membrane of *P. putida* 5-x cell, hence such high adsorption capacity of *P. putida* 5-x cell.

9.4 Advantages of *P. putida* 5-x cell as Biosorbent Compared with Conventional Adsorbent

From section 2.4.10 of chapter 2, we knew that comparison with conventional adsorbent, biosorption technology for removing heavy metal from industrial waste effluent generally have following advantages:

1. Their heavy metals removal capacities are apparently higher than those of conventional adsorbent or ion exchange resin. For example, the uptake of uranium by dead fungal *myelia*, *Rhizopus arrhizus*, has been reported with removal capacity 180 mg U per gram of the sorbent, which was 10-20 times higher than those of commercially available ion exchange resins [Tsezos and Volesky, 1981].

2. Not only the metal uptake capacity of biomass is high, the uptake can also be metal selective [Tobin *et al.*, 1984; Treen-Seavers *et al.*, 1984c]. Usually, the metal of interest was found in a mixture with other metals that may not be required during recovery. Thus, the selectivity of the overall biosorbent process is very important.

3. The biosorbent used may be more economical. Biosorbents might consist of raw biomass, which can be supplied either as waste materials from industrial processes, for example, industrial fermentations and biological wastewater treatment, or as naturally abundant renewable biomaterial like algae and fungi [Volesky, 1987]. It is also possible to propagate the microbial biomass in wastes from various industries. Therefore the biosorbents used could be very economical for detoxifying industrial wastewater solutions.
4. 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\(^{-1}\)) and the effluent must contain less than 1 mg L\(^{-1}\) heavy metals [Tsezos and Keller, 1983; Ross, 1989; Volesky, 1987].

The \textit{P. putida} 5-x cell as biosorbent for Cu\(^{2+}\) and Ni\(^{2+}\) generally also have advantages mentioned above. It is known that in adsorption process, adsorbent regeneration, transport and disposal contributed to major cost compared with cost of adsorbent [Atkinson et al., 1998], thus metal adsorption capacity of adsorbent should be improved as far as possible to reduce the amount of adsorbent used and thus minimizing the sludge regeneration, disposal and transport cost. Thus the high adsorption capacity to heavy metal is the most key of adsorbent.

Compared with conventional adsorbent such resin and activity carbon, the main advantages of \textit{P. putida} 5-x cell were listed as follows:

(1) the adsorption capacity of \textit{P. putida} 5-x cell was obviously high. For example, the Cu\(^{2+}\) adsorption capacity of pretreated cell was about 84 mg g\(^{-1}\), and the cell envelope reached up to 300 mg g\(^{-1}\). However, adsorption capacity of exchange capacity of commercially available activity carbon or ion exchange resins is about 10~50 mg g\(^{-1}\) [Tsezos and Volesky, 1981]. Thus the amount of \textit{P. putida} 5-x cell used in adsorption process will be much less than conventional adsorbent, hence the sludge regeneration, disposal and transport cost is obviously low.

(2) The Cu\(^{2+}\) concentration could be reduced to 1 mg L\(^{-1}\) or below by \textit{P. putida} 5-x cell as adsorbent. However, using conventional adsorbent or resin, the concentration of heavy metal was difficult to be reduced to 1 mg L\(^{-1}\) below.

(3) If organic wastewater was used as culture medium, the production cost of \textit{P. putida}
5-x cell as biosorbent would be lower than resin and activity carbon.

(4) The *P. putida* 5-x cell or its components are biodegradable. When the *P. putida* 5-x cell as biosorbent could not be reused, the cell sludge could be degraded. However, used exchange resin and activity carbon could not be biodegraded. The treatment of used exchange resin and activity carbon is much more difficult than treatment of wastewater.

9.5 Application Area of *P. putida* 5-x Cell as Biosorbent for Cu²⁺ and Ni²⁺ Removal from Wastewater

Although the *P. putida* 5-x cell with high adsorption capacity to Cu²⁺ and Ni²⁺ was isolated from electroplating effluent, and was used to remove and recover the Cu²⁺ and Ni²⁺ efficiently from electroplating effluent, however, the biosorbent *P. putida* 5-x cell also could be used to remove and recover Cu²⁺ and Ni²⁺ from other wastewater. Because when the bacterium *P. putida* 5-x grew on agar medium containing Cu²⁺ or Ni²⁺, the precipitate of black haloes would form around the bacterial colony when the H₂S was sprayed on medium surface. This result indicated that the bacterium *P. putida* 5-x exhibited a high Cu²⁺ and Ni²⁺ accumulating capability in medium containing Cu²⁺ and Ni²⁺, thus apart from electroplating effluent, it certainly can be used to adsorb Cu²⁺ and Ni²⁺ from other Cu²⁺ and Ni²⁺ contained wastewater if the toxicity of other wastewater to the bacterial cell was not considered.

In fact, in this project, the dead cell (or pretreated cell) was used to remove and recover Cu²⁺ and Ni²⁺ from wastewater, thus the toxicity of wastewater to cell biomass, and the effect of wastewater composition on cell growth could not be considered. This is the major advantage of dead cell used as biosorbent compared with living cell. The major
mechanisms of Cu$^{2+}$ and Ni$^{2+}$ adsorption by pretreated P. putida 5-x cell are physical adsorption, precipitation and complexation, not is metabolism-dependent bioaccumulation. According to above discussions, it could be thought that pretreated P. putida 5-x cell could not only be used to remove Cu$^{2+}$ and Ni$^{2+}$ from electroplating effluent, but also to remove Cu$^{2+}$ and Ni$^{2+}$ from other Cu$^{2+}$ and Ni$^{2+}$ contained wastewater, such as metal finishing process industrial wastewater, plastic industrial wastewater and battery or paints industrial wastewater, certainly including these industrial wastewater in overseas, such as Pearl River Delta and other parts of Mainland China). However, in this thesis, the presence of Pb$^{2+}$ inhibited Cu$^{2+}$ and Ni$^{2+}$ adsorption was found, but in electroplating effluent, the concentration of Pb$^{2+}$ is very low, thus the Cu$^{2+}$ and Ni$^{2+}$ could be removed efficiently by P. putida 5-x cell biomass. If the Pb$^{2+}$ concentration in other industrial wastewater would be very high, the adsorption of Cu$^{2+}$ and Ni$^{2+}$ by P. putida 5-x cell might be inhibited. In these cases, the Pb$^{2+}$ should be prior removed, and then Cu$^{2+}$ and Ni$^{2+}$ could be adsorbed efficiently by P. putida 5-x cell. Although the data on adsorption of Pb$^{2+}$ by the P. putida 5-x cell was not listed in this thesis, the adsorption capability of the P. putida 5-x cell to Pb$^{2+}$ was also found during the experiments. About adsorption capability of P. putida 5-x cell to other heavy metals such as Pb$^{2+}$ will be discussed in following section.

9.6 Adsorption Capability of P. putida 5-x Cell to Other Heavy Metal Ions

In this project, the removal and recovery of Cu$^{2+}$ and Ni$^{2+}$ from electroplating effluent was studied. Because, in Hong Kong, Cu$^{2+}$ and Ni$^{2+}$ are the most common heavy metals found in electroplating effluent, and their concentrations are relatively high [Chiu et al., 1987]. The Pb$^{2+}$ or Zn$^{2+}$ are few found or at trace concentration in electroplating effluent. The bacterium P. putida 5-x cell was selected based on the adsorption capacity of Cu$^{2+}$.
and Ni\(^2+\) using plate screening method, however, it does not certainly mean that bacterium *P. putida* 5-x cell has not capability of adsorbing other heavy metal ions. The data on adsorption capability to Pb\(^2+\) and Zn\(^2+\) by *P. putida* 5-x cell was not listed in this thesis, however, the inhibition of Pb\(^2+\) and Zn\(^2+\) to adsorption of Cu\(^2+\) and Ni\(^2+\) by *P. putida* 5-x cell was found during the experiment. For example, when the presence of Pb\(^2+\) in Cu\(^2+\) and Ni\(^2+\) containing wastewater, the Cu\(^2+\) and Ni\(^2+\) adsorption by *P. putida* 5-x was inhibited, however, when the presence of Zn\(^2+\) in wastewater, the Cu\(^2+\) adsorption was not affected obviously, while Ni\(^2+\) adsorption was notably inhibited. In this project, the dead cell or pretreated cell was used as biosorbent, the major adsorption mechanism was physical adsorption, precipitation, ion exchange and complexation. The adsorption sites on cell surface are some electro-negative charged groups. The stronger inhibition of Pb\(^2+\) to Cu\(^2+\) and Ni\(^2+\) indicated that the Pb\(^2+\) exhibited stronger association with electro-negative charged ligands on cell surface than with Cu\(^2+\) and Ni\(^2+\). The presence of Zn\(^2+\) inhibited Ni\(^2+\) adsorption, but not inhibited Cu\(^2+\) adsorption by *P. putida* 5-x cell indicated that Zn\(^2+\) exhibited stronger association with electro-negative charged ligands than Ni\(^2+\), and weaker interaction than Cu\(^2+\). The result implied that the stability of complexes formation of Pb\(^2+\), Zn\(^2+\), Cu\(^2+\) and Ni\(^2+\) with binding sites on cell surface of *P. putida* 5-x cell was in order of Pb\(^2+\) > Cu\(^2+\) > Zn\(^2+\) > Ni\(^2+\). 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\(^2+\) < Zn\(^2+\) < Cu\(^2+\) < Pb\(^2+\) [Hubbeey, 1983]. As shown by the series, both Ni\(^2+\) and Zn\(^2+\) form less stable complexes with electronegative ligands than Cu\(^2+\) and Pb\(^2+\) do. Thus only the presence of Pb\(^2+\) reduced the Cu\(^2+\) removal capacity by 3 folds, while the presence of Cu\(^2+\), Zn\(^2+\) and Pb\(^2+\) all obviously inhibited the Ni\(^2+\) adsorption capacity. The strong inhibition of other heavy metal ions to Cu\(^2+\) and Ni\(^2+\) was due to their high
electropositivity thus strong association with electro-negative ligands, (the electropositivity of lead is 2.33 and that of copper is 1.90) [Hubeey, 1983], so the biosorbent should have higher adsorption capacity to heavy metal ions with stronger electropositivity. According to our experimental results and these discussions, it could be considered that the adsorption capability of \( P. \ putida \) 5-x cell to \( \text{Pb}^{2+}, \text{Cu}^{2+}, \text{Zn}^{2+}, \text{Ni}^{2+} \) should be in order of \( \text{Pb}^{2+} > \text{Cu}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+} \).

In fact, in our experiments on effect of other heavy metal ions on adsorption capacity of \( \text{Cu}^{2+} \) and \( \text{Ni}^{2+} \) by \( P. \ putida \) 5-x cell, the adsorption capacity of \( \text{Pb}^{2+} \) and \( \text{Zn}^{2+} \) was tested by the way. The adsorption capacity of pretreated \( P. \ putida \) 5-x cell to \( \text{Pb}^{2+} \) and \( \text{Zn}^{2+} \) was about 190 mg g\(^{-1}\) and 75 mg g\(^{-1}\), respectively, and the cell envelope of \( P. \ putida \) 5-x cell to \( \text{Pb}^{2+} \) and \( \text{Zn}^{2+} \) was about 480 mg g\(^{-1}\) and 270 mg g\(^{-1}\). Obviously, the pretreated \( P. \ putida \) 5-x cell or its cell envelope could also be used as biosorbent for \( \text{Pb}^{2+} \) and \( \text{Zn}^{2+} \).

However, \( \text{Pb}^{2+} \) and \( \text{Zn}^{2+} \) are in trace, and both \( \text{Ni}^{2+} \) and \( \text{Cu}^{2+} \) are predominant in electroplating effluents of Hong Kong. Thus in this project, the adsorption capacity and characteristics of \( P. \ putida \) 5-x cell to \( \text{Cu}^{2+} \) and \( \text{Ni}^{2+} \) was studied.

If other industrial wastewater containing a large amount of \( \text{Pb}^{2+} \) or \( \text{Zn}^{2+} \) should be treated, the \( P. \ putida \) 5-x cell also could be used to remove \( \text{Pb}^{2+} \) or \( \text{Zn}^{2+} \) from wastewater. However, certainly, the optimal \( P. \ putida \) 5-x cell production condition as biosorbent for \( \text{Pb}^{2+} \) or \( \text{Zn}^{2+} \), the optimal adsorption conditions and characteristics, and the inference of other heavy metal ions to \( \text{Pb}^{2+} \) or \( \text{Zn}^{2+} \) adsorption should be further studied.

9.7 Advantages of Bacterial Cell as Biosorbent Compared with Other Microorganism Cell

Biosorption is the sorption and/or complexation of dissolved metals based on the
chemical activity of microbial biomass. The major adsorption sites on microbial biomass are some electro-negative charged groups. From Figure 2.4, 2.6 and 2.8, it could be found that all of cell surface, such as algae, fungi, bacteria was made up of some components such as mucilage, cell wall, plasma membrane, outer membrane and inner membrane. These components are composed of some organic materials such as proteins, phospholipid, lipopolysaccharide, PEG, chitin fibres, alginate, cellulose fibres and so on, which containing some electro-negative charged groups. Therefore they could interact with cationic metallic species, and thus could be used as biosorbent for heavy metal ions adsorption. In previous, many studies reported that algae, fungi, bacteria and their components were used as biosorbent to remove and recover heavy metal ions from wastewater successfully. However, compared with other microorganisms, bacterial cells have some advantages as follows:

(1) the surface area per volume of bacterial cell is much high due to its small volume, thus heavy metal ions adsorbed on per volume is much more than alga and fungi cell.

(2) compared with algae and fungi cells, the growth rate of bacterial cells are more quick, thus the culture time of bacterial cell is shorter than algae and fungi cell, hence, the production cost of bacterial cells is low.

(3) the culture conditions of bacterial cell is relative simple than algae and fungi.

(4) the bacterial cell could be simply immobilized on medium for its efficient separation from wastewater, thus can be regenerated and reused efficiently.

(5) in practical application, many by-products of fermentation industries and activity sludge can be used as biosorbent, in which bacterial cell are predominant generally.

Thus, in this study, a gram-negative bacterial cell P. putida 5-x with high Cu$^{2+}$ and Ni$^{2+}$ adsorption capacity was selected as biosorbent to remove and recover Cu$^{2+}$ and Ni$^{2+}$ from
industrial wastewater. The objective of this project is to study the adsorption capacity, adsorption conditions, adsorption kinetic and adsorption mechanism of gram-negative bacterial cell as biosorbent for providing a general indication in using gram-negative bacterial cell as biosorbent to remove and recover heavy metal ions from wastewater in practical application.
Chapter 10: Conclusions and Recommendations

10.1 Conclusions

The present study mainly focus on to develop an inexpensive and effective biosorption process for removing and recovering Cu$^{2+}$ and Ni$^{2+}$ from local electroplating effluent, and to study the heavy metal adsorption mechanism by bacterial cell. For the objective, in the project, bacteria with high adsorption capacity to both Cu$^{2+}$ and Ni$^{2+}$ were isolated and selected. Optimal bacterial cell production technique as biosorbent for Cu$^{2+}$ and Ni$^{2+}$, and optimal adsorption/desorption(recovery) condition were studied. In addition, cell immobilization technology, biosorption reactor, and adsorption characteristics and mechanisms also were investigated. The major results obtained in the study are summarised below:

1. A bacterium with high Cu$^{2+}$ and Ni$^{2+}$ accumulating capability was isolated from electroplating effluent, which was tentatively identified as a gram-negative Pseudomonas Putida, and was named as Pseudomonas Putida 5-x.

2. For Cu$^{2+}$ and Ni$^{2+}$ binding, the cell cultured in sulfate and ammonia limiting medium had evidently high adsorption capacity compared with that in other media, this might be due to the accumulation of large amount of phospholipids and polysaccharides when cell cultured in sulfate and ammonia limiting medium. However, owing to ammonia limiting medium had an obvious negative effect on cell growth, the sulfate limiting medium was thus used for efficient cell production as biosorbent for Cu$^{2+}$ and Ni$^{2+}$.

3. Cu$^{2+}$ and Ni$^{2+}$ adsorption capacity of P. putida 5-x cell in different cell growth phases were various. Both TEM and X-ray analysis confirmed the phenomenon. The bacterial cells harvested in 34-38 h and 28-30 h had maximum Cu$^{2+}$ and Ni$^{2+}$ adsorption capacity,
respectively. However, considering both cell mass and adsorption capacity, the cells harvested in 34~36 h and 30~32 h was optimal cells as biosorbent for Cu$^{2+}$ and Ni$^{2+}$ adsorption, respectively.

4. Experimental results indicated that higher incubation temperature had a negative effect on Cu$^{2+}$ and Ni$^{2+}$ adsorption of P. putida 5-x cell, the effect was particularly obvious to the cell in logarithmic growth phase. This might be due to high temperature inhibits the formation of some active matter heavy metal binding related.

5. Cell pretreatment using diluted HCl could increase Cu$^{2+}$ adsorption capacity by 24% and Ni$^{2+}$ adsorption capacity by 31%, respectively, with low cell biomass loss rate. TEM analysis indicated that enhanced adsorption of Cu$^{2+}$ and Ni$^{2+}$ by pretreated cells was relative to the degradation of a loose superficial layer outside the fresh cell - capsule.

6. Adsorption kinetic study showed that the adsorption of Cu$^{2+}$ and Ni$^{2+}$ by fresh cell consisted of two phases, namely a rapid, metabolism-independent metal ion adsorption phase (biosorption) followed by a slow bioaccumulation phase. Pretreated cell only consisted of a rapid adsorption phase. About 80% of the total Cu$^{2+}$ and Ni$^{2+}$ taken up by the fresh bacterial cells were removed within the rapid adsorption phase.

7. Glucose and sodium azide test indicated that the adsorption of fresh cell in rapid phase hardly was metabolism-dependent. However, the adsorption in slow bioaccumulation phase of fresh cell seemed to be metabolism-dependent. Adsorption of pretreated cell was metabolism-independent.

8. pH obviously affected Cu$^{2+}$ and Ni$^{2+}$ adsorption capacity of P. putida 5-x cell. The high the pH, the high the Cu$^{2+}$ and Ni$^{2+}$ adsorption capacities. This is because that (1) hydrogen ions act as competition ion against Cu$^{2+}$ and Ni$^{2+}$ binding, (2) at low pH solution, some active groups heavy metal binding related are electropositive. Only at
higher pH solution these active groups change to electronegative for efficiently binding heavy metal

9. Other cations such as Pb$^{2+}$, Zn$^{2+}$ could act as competition ions to affect Cu$^{2+}$ and Ni$^{2+}$ adsorption. Pb$^{2+}$ appeared strong inhibition to Cu$^{2+}$ adsorption, but Zn$^{2+}$ and Ni$^{2+}$ only little. However, Pb$^{2+}$, Cu$^{2+}$ and Zn$^{2+}$ all appeared strong inhibition to Ni$^{2+}$ adsorption. The affinity of *P. putida* 5-x cell biomass to Pb$^{2+}$. Cu$^{2+}$, Zn$^{2+}$ and Ni$^{2+}$ was in order of Pb$^{2+} >$ Cu$^{2+} >$ Zn$^{2+} >$ Ni$^{2+}$. When mole concentration of Cu$^{2+}$: Ni$^{2+}$ in wastewater was 0.1 below, the inhibition of Cu$^{2+}$ to Ni$^{2+}$ adsorption of *P. putida* 5-x cell disappeared basically. Anions such as Cl$^-$, SO$_4^{2-}$ and NO$_3^-$ hardly inhibited the Cu$^{2+}$ and Ni$^{2+}$ uptake. However, EDTA obviously inhibited both Cu$^{2+}$ and Ni$^{2+}$ uptake.

10. The Cu$^{2+}$ adsorption process of fresh and pretreated cell within the experimental range could be expressed by *Freundlich* isotherm of $Q_p = 26.9 \ C_e^{0.73}$ and $Q_f = 12.8 \ C_e^{0.64}$, respectively, while Ni$^{2+}$ adsorption could be expressed as $Q_p = 18.2 \ C_e^{0.21}$ and $Q = 10.9 \ C_e^{0.265}$, respectively. This illustrated that Cu$^{2+}$ and Ni$^{2+}$ binding on fresh and pretreated cell surface could be considered as adsorption process. Adsorption isotherms showed that pretreated cell was a better biosorbent either for Cu$^{2+}$ or for Ni$^{2+}$ than fresh cell. In addition, experiments found that in higher pH solution, Cu$^{2+}$ or Ni$^{2+}$ adsorption process of *P. putida* 5-x cell obeyed the *Freundlich* isotherm, but in lower pH solution, the adsorption seemed to be better described with *Langmuir* isotherm.

11. 0.1-0.3 M HCl was chosen to be better desorption solution for Cu$^{2+}$ and Ni$^{2+}$ recovering from loaded biosorbent. 96 % and 99 % above bound Cu$^{2+}$ and Ni$^{2+}$ could be desorbed by this desorption solution only with 3-6 % of biomass loss rate.

12. In view of both recovery rate and heavy metal concentration in desorbed solution points, 50 ml:1000 mg of 0.1 M HCl : loaded biosorbent was a better volume ratio. In this volume
ratio, 96% and 99% above recovery rate could be attained with Cu²⁺ and Ni²⁺ concentration of 1.63 g L⁻¹ and 0.75 g L⁻¹ in desorbed solution, respectively.

13. Desorption kinetic study indicated that Cu²⁺ and Ni²⁺ desorption process from loaded biosorbent was quite rapid. 95% Cu²⁺ and 99% Ni²⁺ was desorbed before first 5 minutes with less biomass loss rate.

14. *P. putida* 5-x cell as biosorbent could be effectively regenerated and reused at least five cycle for removing and recovering Cu²⁺ or Ni²⁺ with adsorption capacity of around 84 mg g⁻¹ or 37 mg g⁻¹. The desorption rate also reached 96% for Cu²⁺ and 99% for Ni²⁺.

15. Magnetite was chosen to immobilize *P. putida* 5-x cell for improving its sedimentation, mechanical strength and rigidity. Experiment results showed that magnetite alone also possessed the Cu²⁺ and Ni²⁺ adsorption capability, but magnetite immobilized *P. putida* 5-x cell appeared much higher Cu²⁺ and Ni²⁺ adsorption capacity compared with magnetite alone. The Cu²⁺ and Ni²⁺ adsorption by both magnetite alone and magnetite immobilized cells obeyed the *Freundlich* isotherms of $Q_{M-Cu} = 2.1 C_{e-Cu}^{0.68}$, $Q_{M-Cu} = 11.9 C_{e-Cu}^{0.74}$ and $Q_{M-Ni} = 0.78 C_{e-Ni}^{0.79}$, $Q_{M-Ni} = 9.7, C_{e-Ni}^{0.21}$.

16. A semi-continuous stirred reactor with magnetite-immobilized *P. putida* 5-x cell as biosorbent was used to remove and recover Cu²⁺ (or Ni²⁺) from Cu²⁺ (or Ni²⁺) containing wastewater. In suitable condition, 97% above Cu²⁺ (or Ni²⁺) removal efficiency could be obtained by this system, and the Cu²⁺ (or Ni²⁺) concentration in treated effluent was 1.0 mg L⁻¹, below. The recovery rate of Cu²⁺ (or Ni²⁺) from loaded immobilized cell reached 96% (or 98%) above, while the Cu²⁺ (or Ni²⁺) concentration in desorbed solution reached 0.71 g L⁻¹ (or 0.72 g L⁻¹). The *P. putida* 5-x cell immobilized on magnetite played the most important parts in the adsorption process. The immobilized cell could be effectively regenerated and reused five cycles at least.
17. A two-stage semi-continuous stirred reactor with immobilized *P. putida* 5-x cell as biosorbent was developed to sequentially remove and recover Cu\(^{2+}\) and Ni\(^{2+}\) from wastewater. In suitable operation conditions, the two-stage system could effectively remove and recover Cu\(^{2+}\) and Ni\(^{2+}\) sequentially from synthetic wastewater at least five cycles. The Cu\(^{2+}\) and Ni\(^{2+}\) removal efficiency of 96 % and 97 % could be attained, while their concentration in treated effluent reduced from 30 mg L\(^{-1}\) to around 1 mg L\(^{-1}\) for Cu\(^{2+}\) and 0.9 mg L\(^{-1}\) below for Ni\(^{2+}\). The Cu\(^{2+}\) and Ni\(^{2+}\) recovery rate from loaded biosorbent could reach 95 % and 98 % above, respectively, while the Cu\(^{3+}\) and Ni\(^{2+}\) concentration in each desorbed solution reached 0.68 g L\(^{-1}\) and 0.7 g L\(^{-1}\) above. Although the treatment efficiency of the two-stage biosorption system to real electroplating wastewater was slightly lower than to synthetic wastewater due to the interfering of other cations and anions in real wastewater, the Cu\(^{2+}\) and Ni\(^{2+}\) concentrations in the treated effluent also basically met the effluent discharge standard of Environmental Protection Department of Hong Kong.

18. The removal of cell superficial layer-capsule by 0.1M HCl enhanced the Cu\(^{2+}\) adsorption of *P. putida* 5-x cell. The Cu\(^{2+}\)-bridging formed by binding divalent Cu\(^{2+}\) on electronegative groups in capsule might induce a conformation change within the capsule, thus resulted in some metal-binding sites on cell outer membrane or PEG layer inaccessible for Cu\(^{2+}\) binding.

19. Separated cell envelopes increased 4 times more Cu\(^{2+}\) adsorption capacity than that of fresh intact cells. The total amount of Cu\(^{2+}\) bound by 2.41 g separated cell envelopes isolated from 6g fresh cell was 1.4 times more than by that of 6 g fresh cells. This indicated that chemical and physical treatment during the separation of cell envelopes liberated more metal-binding sites on cell envelopes becoming heavy metal accessible.

20. Adsorption experiments indicated that separated PEG layer materials, outer
membrane and inner membrane all played roles on Cu$^{2+}$ adsorption by cell envelope of *P. putida* 5-x. The Cu$^{2+}$ adsorption capacity of PEG layer, outer membrane and inner membrane were in order of PEG>outer membrane > inner membrane, but their content in cell envelope was in order of inner membrane> outer membrane>PEG layer. Considering both adsorption capacity and content of cell surface components, the total contribution of PEG layer, outer membrane and inner membrane to Cu$^{2+}$ adsorption of the cell envelopes was in order of outer membrane > inner membrane > PEG layer materials.

21. The variation of Cu$^{2+}$ adsorption capacity of cell envelopes in different cell growth phases was due to the content variation of PEG layer, outer membrane and inner membrane in cell envelope, and the variation of Cu$^{2+}$ adsorption capacity of outer membrane and inner membrane in different cell growth phases. Particularly, the content variation of PEG layer and outer membrane, and the Cu$^{2+}$ adsorption capacity variation of outer membrane in different cell growth phases played an important part to the variation of Cu$^{2+}$ adsorption capacity of cell envelopes in different cell growth phases.

22. The content variation of both phospholipids and lipopolysaccharides in outer membrane resulted in the variation of Cu$^{2+}$ adsorption capacity of outer membrane in different cell growth phases, while the content variation of only phospholipids in inner membrane resulted in the variation of its Cu$^{2+}$ adsorption capacity in different cell growth phases.

### 10.2 Limitations and Recommendations

The complexity of the structure of gram-negative bacterium implies that there are many ways for the metal to be captured by the cell. Therefore, mechanisms of biosorption of *P. putida* 5-x cell may be not constant in different cases. In this project, the study on role
and contribution of cell surface components to heavy metal binding of *P. putida* 5-x cell was not overall, the experimental results were obtained only in certain experimental condition. However, in industrial application, the cell culture conditions and adsorption conditions are more various, thus, the adsorption mechanism and adsorption properties should be further studied in various rigorous conditions. For example, the Cu\(^{2+}\) and Ni\(^{2+}\) removal efficiency of magnetite immobilized *P. putida* 5-x cell to real electroplating was lower than to synthetic wastewater, this illustrated that some reasons affected the Cu\(^{2+}\) and Ni\(^{2+}\) adsorption of *P. putida* 5-x cell are not still clarified clearly. If the adsorption properties and adsorption mechanism in rigorous industrial circumstance can be further clarified, suitable adsorption condition will be designed to effectively enhance treatment efficiency of *P. putida* 5-x cell to Cu\(^{2+}\) and Ni\(^{2+}\) in practical application. Indeed, more information and evidences could be provided by conducting experiments such as solid nuclear magnetic resonance spectroscopy (solid-NMR), X-ray absorption fine structure spectroscopy (EXAFS), and so on.

Although the use of magnetite as the support matrix for cell immobilization was proved to be suitable for laboratory-scale model systems, the treatment efficiency of the magnetite immobilized cell should be further evaluated in large-scale process. The further selection of other much better immobilization supports that possesses high mechanical and chemical stability with no decrease in the metal removal capacities are also recommended.

Mathematical models play a key role in transferring technologies from the laboratory to a full-scale application. Good models can not only help in analysing and interpreting experimental data but also in predicting the performance of the biosorption process under different operating conditions. Hence, analysis of biosorption performance has to be described by more different models such as ion exchange and surface complexation.
models. Langmuir and Freundlich adsorption models may be too simple in describing the experimental data since they do not take into account of solution pH change and the presence of other ions in the solution system.

To be of practical use, an appropriate bioreactor design should be developed for evaluating industrial-scale biosorption performance, based on data from laboratory-scale experiments. The cost efficiency, ease of use and size of the engineered system are the key factors for engineering considerations. Also, portability is also important for some industrial applications.
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