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A Novel Magnetite-Immobilized Cell Process for Heavy Metal Removal from Industrial Effluent

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Abstract

The sorption and desorption of copper (II) (Cu[II]) ions from the wastewater by magnetite-immobilized cells of *Pseudomonas putida 5-x* with acidic pretreatment were studied. Pretreating cells with 0.6 *N* HCl was found to enhance greatly the adsorption capacity of biomass up to 85.6 mg/g and had no significant effect on the loss of *P. putida 5-x* cells during biosorbent pretreatment. The biosorption capacity to Cu^{2+} of magnetite-immobilized cells of *P. putida 5-x* harvested during various growth phases was also investigated. The experimental results illustrated that the adsorption capacity to Cu^{2+} of *P. putida 5-x* cultured in sulfate-limiting medium reached maximum during the late stationary growth phase or early death phase, and reached minimum during the log growth phase. The mechanism of copper sequestering by this type of biomass was studied via transmission electron microscopy. A degradation of the peptidoglycan layer of the cell wall was observed in the acidic pretreatment, but no further degradation appeared after the adsorption-desorption cycle. Cu(II) accumulated mostly on the surface of the cell walls and was effectively desorbed by the acidic treatment during the desorption process.

Index Entries: Magnetite-immobilized cells; copper; sorption and desorption mechanism; transmission electron microscopy analysis; growth phase; *Pseudomonas putida 5-x*.

Introduction

The toxicity of copper to humans and microorganisms has been well documented *(1)*. In Hong Kong, copper pollution arises mainly from the

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finishing and electroplating industries *(2)*. Unlike organic pollutants, which in most cases can eventually be destroyed, copper species released into the environment persist indefinitely and eventually enter the food chain, thus causing harm to humans *(3,4)*. Therefore, copper ions must be removed from industrial waste effluent. On the other hand, copper is valuable and can be recovered for reuse.

The most common methods for removing metals include chemical precipitation, chemical oxidation and reduction, ion exchange, filtration, electrochemical treatment, and evaporative recovery. However, these procedures have significant disadvantages: they remove metal incompletely, have high reagent requirements, generate toxic sludge, and are quite expensive when the contaminant concentrations are lower than 100 mg/L *(5)*.

During the past decade, biosorption of toxic metals as an alternative technology for removing heavy metals has received much attention *(6)*. Metals and microbial cells can interact through adsorption to cell surfaces and accumulate within the cells as metal complexes. Bacteria, algae, and fungi have been used successfully as adsorbing agents for copper ions; bacteria are of particular interest because their surface:volume ratio is often relatively high, especially in the case of nonspherical forms *(7,8)*. For biosorption to function efficiently in more rigorous industrial applications, the microbial biomass should be immobilized in a particulate form, which preserves the biomass biosorptive properties and provides physical characteristics similar to those of conventional adsorbent particles such as activated carbon or ion-exchange resins *(9)*. A proprietary immobilization technique has been developed; the magnetite immobilization technique seems to be the most attractive *(10–12)*. 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.

The bacterial strain *Pseudomonas putida 5-x*, isolated from electroplating effluent, has been tested for magnetite-immobilized biosorption study. The characteristics of magnetite-immobilized cells of *P. putida 5-x* for removing copper, such as kinetics of copper uptake, biosorption capacity, and the effects of pH, cations, and anions, have been well documented *(13)*. However, some problems remain. First, the effect of pretreating biomass on the biosorbent efficiency is still unknown. Metal removal can be significantly increased depending on the pretreatment step *(14)*. For example, acid treatment may be able to remove impurities such as potassium from the biomass. Potassium is present in the growth medium, and may be immobilized on the biomass surface; removal of potassium during pretreatment will therefore free metal binding sites for uptake of more metal ions *(15)*. Second, it is necessary to find an optimal harvest period of

P. putida 5-x cultured in sulfate-limiting medium (SLM), which will yield numerous cells with optimal adsorption capacity to Cu^{2+}. The biosorption capacity of heavy metal was mainly relative to the surface structure of the cell *(10)*, and the adsorption activity centers of the cell surface to heavy metal are some protein or carboxylate groups *(16,17)*. There are five phases of microorganism growth: lag, accelerated growth, log growth, stationary growth, and death. The surface structure and features of bacterial cells vary in each, so the heavy metal adsorption capacity of bacterial cells may differ in various growth phases. However, a few studies have focused on that aspect. Third, understanding of the mechanism of copper uptake on the *P. putida 5-x* cells is still in its infancy. For immobilized biomass, the biosorbent can be reactivated and the loaded metals recovered after desorption in a manner similar to ion-exchange resins. However, the biosorption mechanisms, and hence the desorption mechanisms, for biosorbent are much more complicated than those for ion-exchange resins. Factors such as type of biosorbent, type and concentration of desorbent, and type of metal ions can affect desorption efficiency *(18)*.

The objectives of the present study were to investigate the effect of acidic pretreatment of magnetite-immobilized cells of *P. putida 5-x* on the removal and recovery of copper from the wastewater, to study the difference between adsorption capacity in various growth phases of *P. putida 5-x*, and to study the mechanism of copper uptake by analyzing the Cu(II) accumulation in *P. putida 5-x* in an adsorption-desorption procedure.

Materials and Methods

Microorganisms, Growth Conditions, and Pretreatment

The bacterial strain used in this study, *P. putida 5-x* was isolated from the local electroplating industrial effluents. The microbes were grown in Luria-Bertani medium, mixed with glycerol (final concentration 25%), and stored at –70°C. The bacterial inoculum was prepared by inoculating a single colony in 5 mL of SLM in a test tube and incubated at 30°C with shaking at 200 rpm for 24 h *(10)*. The SLM, which contained 1% inoculum, was then incubated at 30°C with shaking at 200 rpm for 48 h. The microbes were harvested by centrifugation at 12,000 rpm for 10 min at 4°C. The cell pellets were washed twice using 100 mL of 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer *(19)*. For the acidic pretreatment, 100 mg (dry wt) of *P. putida 5-x* biomass sample was washed with 25 mL of dilute acids (HCl, H_2SO_4, or $[NH_4]_2SO_4$) with shaking at 200 rpm for 30 min at 25°C and then washed with 50 mL of MES buffer.

Preparation of Magnetite-Immobilized Cells of P. putida 5-x

The bacterial cells were immobilized using the following procedure. Fifty milligrams (dry wt) of pretreated or fresh *P. putida 5-x* cells was suspended in 50 mL of MES buffer solution at pH 5.5. Then 250 mg of magnetite

(Sigma-Aldrich, St. Louis, MO) was added to the suspension. The mixture was shaken in a rotary shaker at 200 rpm for 5 min at 20–25°C. The microbial cells and magnetite mixtures settled after 5 min with the aid of an electromagnet. More than 99.0% of bacterial cells could be immobilized on magnetite (microscope test) using this method.

Adsorption Capacity of Copper from Copper Solution

Twenty-five milligrams of *P. putida 5-x* cells immobilized on 125 mg of magnetite was added separately into 100 mL of aqueous solution with various copper concentrations (4, 40, 100, and 180 mg/L). The mixtures were then shaken at 200 rpm in rotary shakers for 30 min at 25°C. The adsorption capacities of the mixtures were determined by measuring the concentrations of copper in the supernatant after settling.

Removal and Recovery of Copper from Wastewater

Trials for removing and recovering copper ions from wastewater were performed in a system comprising a series of three batch biosorption reactors (Fig. 1). Each reactor consisted of a 500-mL glass beaker, a magnetic stirrer, and an electromagnet. The magnetic stirrer was operated at 200 rpm. Separate stirrers were used for the sorption and settling phases. The Cu(II) bearing wastewater was fed countercurrently to the biosorbent into the reactor to obtain the lowest possible copper concentration in treated wastewater and the highest possible copper concentration in the biosorbent. Removed copper and biosorbent were passed through a 0.4-μm filter and then recovered in an acid regeneration reactor. The regenerated biosorbent was reused in the first biosorption reactor after being washed with the MES buffer. The constituents and operating conditions of the acid regeneration reactor and buffer wash tank were the same as those of the biosorption reactors.

Transmission Electron Microscopy Analysis

Cell-magnetite-Cu(II) complexes from adsorption-desorption processes and magnetite-free cells not previously exposed to Cu(II) were examined as whole mounts and thin sections with a JEM-1200 EX-II TEM (JEOL, Tokyo, Japan), which was operated at 80 kV under standard conditions. For whole mounts, carbon-Formvar-coated nylon grids (200 mesh, 3 mm od) (Pelco, Redding, CA) were floated in aqueous cell suspensions for 10 s, dried with blotting paper, and examined. For thin sections, the sample pellets were harvested and concentrated by centrifugation, fixed with 2.5% glutaraldehyde in 0.2 *M* phosphate buffer solution (phosphate-buffered saline [PBS], pH 7.0) at 4°C for 2 h, and washed twice with PBS for 10 min. An acetone series (30, 50, 70, 95, and 100% acetone) was used in the dehydration procedure, in which the samples were treated for 10 min with each concentration of acetone. The samples were infiltrated with acetone: Spurr mixtures (Spurr materials; EMS, Fort Washington, PA) with ratios of

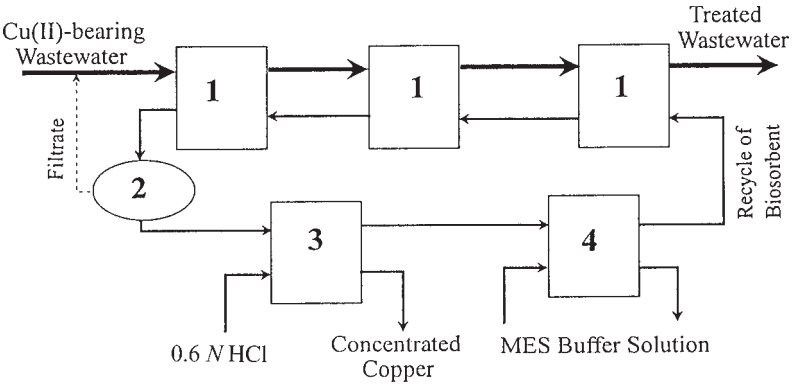

Fig. 1. Schematic diagram of treatment of Cu(II)-bearing wastewater by *P. putida 5-x* immobilized on magnetites. 1, Biosorption reactor; 2, filter; 3, acid regeneration reactor; 4, buffer wash tank.

1:1 and 1:2, each for 2 h, and kept overnight in the 1:2 acetone:Spurr mixture. The samples were then treated with 100% Spurr twice, each for 2 h, and then embedded in Spurr at 68°C for 16 h. Thin sections of 60 nm were prepared using a Reichert Ultracuts (Leica, Wien, Austria) equipped with a diamond knife (Diatome 45°, Fort Washington, PA). Sections were collected on carbon-Formvar-coated nylon grids and stained with and without a 2% (w/v) uranyl acetate and lead citrate solution, respectively, for 10 min. The excess stain was then washed away with deionized water.

Results and Discussion

Effect of Acid Pretreatment on the Uptake of Copper

Comparisons for copper adsorption capacities of fresh *P. putida 5-x* with those of acid-pretreated cells showed that pretreating cells with acid could greatly enhance the adsorption capacity by 7–27% (Table 1). Pretreating *P. putida 5-x* cells removed the impurities in the biomass, which enhanced its adsorption capacity.

Effects of Acid Pretreatment on Biomass Loss

The biomass used as a biosorbent must not have a high biomass loss rate (BLR) in the pretreatment procedure. The effect of pretreatment on the *P. putida 5-x* biomass was studied using 100 mg (dry wt) of *P. putida 5-x* biomass and 25 mL of various chemical solutions. All these solutions could be used as eluents to regenerate biomass. (Table 2 lists the BLRs of various eluting solutions in pretreatment trials) The BLRS were <16% if the HCl concentrations were lower than 0.6 *N* and when $(NH_4)_2SO_4$ solutions were used as the eluent. Other solutions, such as the 0.9 *N* and 1.2 *N* HCl, and H_2SO_4 were not suitable as eluants because of their higher BLRs.

Biomass loss rate was also investigated over five cycles of operation to assess the reusability of the cells after washing with 0.3 *N* and 0.6 *N* HCl

Table 1
Copper Adsorption Capacities of Fresh and Pretreated-Immobilized Cells with Different Acids (mg/g dry wt)

	Fresh cell	0.3 *N* HCl	0.6 *N* HCl	15% $(NH_4)_2SO_4$
Adsorption capacity	67.4	83.5	85.6	72.1
Rate of increase (%)	—	21	27	7

Table 2
BLR (%) with Various Eluent Pretreatment

	HCl					H_2SO_4		$(NH_4)_2SO_4$	
Eluent	0.1 *N*	0.3 *N*	0.6 *N*	0.9 *N*	1.2 *N*	0.1 *N*	0.5 *N*	10%	15%
BLR (%)	14.5	15.2	15.8	19.6	22.3	31.4	35.0	11.5	12.4

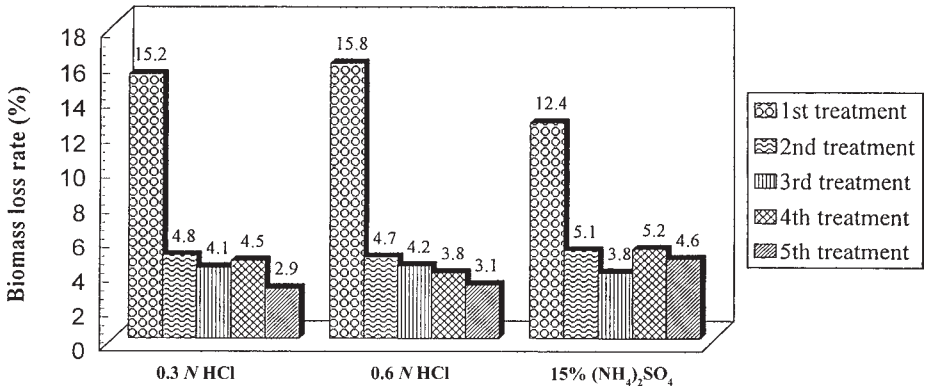

Fig. 2. BLR after using 10 mg of *P. putida 5-x* with 25 mL of various eluents in a rotary shaker at 200 rpm for 30 min.

and 15% $(NH_4)_2SO_4$. As shown in Fig. 2, the BLRs for the tested eluents were much higher during the first wash cycle (about 15%) than those observed during the following wash cycles (<5%). This indicated that the impurities or acid-soluble matter in fresh biomass could be washed away through pretreatment with the same eluent used to regenerate the biosorbent. There was no significant loss of *P. putida 5-x* cells during the biosorbent pretreatment with the same eluent.

Desorption of Copper from Cells

The desorption efficiency using 0.6 *N* HCl eluent was assessed by determining the bound copper to the biosorbent (Table 3). Significantly higher recovery efficiencies were obtained by using 10 mL or more of 0.6 *N* HCl eluent. The Cu(II) concentration in the recovery solution was 1.7 g/L when the recovery efficiency was 99.1%. The 0.6 *N* HCl eluent

Table 3
Recovery of Cu(II) Using 0.6 *N* HCl[a]

Acid volume (mL)	Cu(II) concentration (g/L)	Cu(II) recovery efficiency (%)
5	2.85	83.2
10	1.7	99.1
15	1.13	99.5
20	0.85	99.4

[a]Cu(II) (17.12 mg) was adsorbed by 200 mg *of P. putida 5-x* cells immobilized on 1000 mg of magnetite (85.6 mg of Cu[II] g of dry cell).

effectively recovered copper from the magnetite-immobilized cells of *P. putida 5-x*. The cells could thus be regenerated and reused.

Removal and Recovery of Copper from Wastewater

By operating the three batch biosorption reactors to study Cu(II) removal and recovery, data were collected after the fifth cycle, in which equilibrium was achieved. The effects of adsorption and desorption were investigated over eight cycles to assess the reusability of the cells. The results listed in Table 4 indicate that the average copper recovery rate was higher than 95% for all tests. The Cu(II) concentration in the recovery solution could be as high as 1.4 g/L and was ready to be reused.

Effect of Growth phase of P. putida 5-x *and* Cu^{2+} *Concentration on Adsorption Capacity*

P. putida 5-x was cultured in SLM with shaking at 200 rpm at 25°C. The sample was collected and its growth tested by testing the optical density once every 2 h (Fig. 3A). The growth of *P. putida 5-x* in SLM medium could be divided into five phases: lag phase (0–10 h), accelerated growth phase (12–16 h), log growth phase (18–30 h), stationary growth phase (32–42 h) and death phase (42–48 h). Because the amount of biomass in the lag phase was less, the adsorption capacity and characteristics of bacterial cells to Cu^{2+} in this phase were not studied.

Bacterial cells grown in accelerated growth, log growth, stationary growth, and death phases were harvested at 12, 18, 24, 30, 36, 42, and 48 h, respectively, and the biosorption capacity of magnetite-immobilized *P. putida 5-x* to Cu^{2+} in aqueous solution containing 40, 100, and 180 mg/L of Cu^{2+} was determined (Fig. 3B). During the accelerated growth phase (corresponding to 12–18 h), the adsorption capacity of bacterial cells to Cu^{2+} descended slowly, but when microorganism growth reached log growth phase (corresponding to 18–30 h), the Cu^{2+} biosorption capacity of the bacterial cells decreased sharply. The lowest point of adsorption capacity appeared at 24–26 h (the early or mid log growth phase). When growth reached the late log growth phase, the adsorption capacity of bacterial cells to Cu^{2+} ascended slowly from the bottom and accelerated during the sta-

Table 4
Results of Batch Biosorption Reactor Operation

Cu(II) concentration in wastewater	(mg/L)	100
Batch treatment wastewater volume	(mL)	300
Batch regeneration acid volume	(mL)	20
Circulation biomass weight	(g dry cell)	0.5
After 5th cycle:		
Treated wastewater Cu(II)	(mg/L)	3.42
Concentrated Cu(II)	(g/L)	1.42
After 6th cycle:		
Treated wastewater Cu(II)	(mg/L)	4.25
Concentrated Cu(II)	(g/L)	1.38
After 7th cycle:		
Treated wastewater Cu(II)	(mg/L)	3.14
Concentrated Cu(II)	(g/L)	1.47
After 8th circle:		
Treated wastewater Cu(II)	(mg/L)	2.86
Concentrated Cu(II)	(g/L)	1.44
Average treated wastewater Cu(II)	(mg/L)	3.42
Average concentrated Cu(II)	(g/L)	1.43
Average Cu(II) removal rate[a]	(%)	96.6
Average Cu(II) recovery rate[b]	(%)	95.4

[a][Cu(II) concentration in wastewater-average treated wastewater Cu(II)]/Cu(II) concentration in wastewater.

[b]Average concentrated Cu(II) × batch regeneration acid volume/Cu(II) concentration in wastewater × batch treatment wastewater volume.

tionary growth phase. In the late death phase, the adsorption capacity of *P. putida 5-x* to Cu^{2+} reached maximum. Otherwise, the variation of biosorption capacity of bacterial cells to 40 mg/L of Cu^{2+} was not notable, but was drastic to 180 mg/L of Cu^{2+}. The minimum adsorption capacity was only 21 mg/g at 24 h of log growth phase. But during the late stationary growth and early death phases of *P. putida 5-x* in SLM medium, the adsorption capacity to Cu^{2+} was similar to other Cu^{2+} concentration solutions. These results showed that adsorption capacity to Cu^{2+} of *P. putida 5-x* in other growth phases was different, and that other Cu^{2+} concentrations in aqueous solution affected the adsorption capacity to Cu^{2+} of *P. putida 5-x* in various growth phases, but was not notable during the late stationary growth and early death phases.

Transmission Electron Microscopy Test and Proposed Mechanism

Transmission electron microscopy (TEM) analysis was carried out on the whole mounts of air-dried cells of *P. putida 5-x*. TEM enables the comparison the cell wall appearance before and after acidic pretreatment. The cell wall and a plasma membrane outside the cell wall of *P. putida 5-x* were examined by TEM, but after the cell was pretreated with 0.6 *N* HCl, that

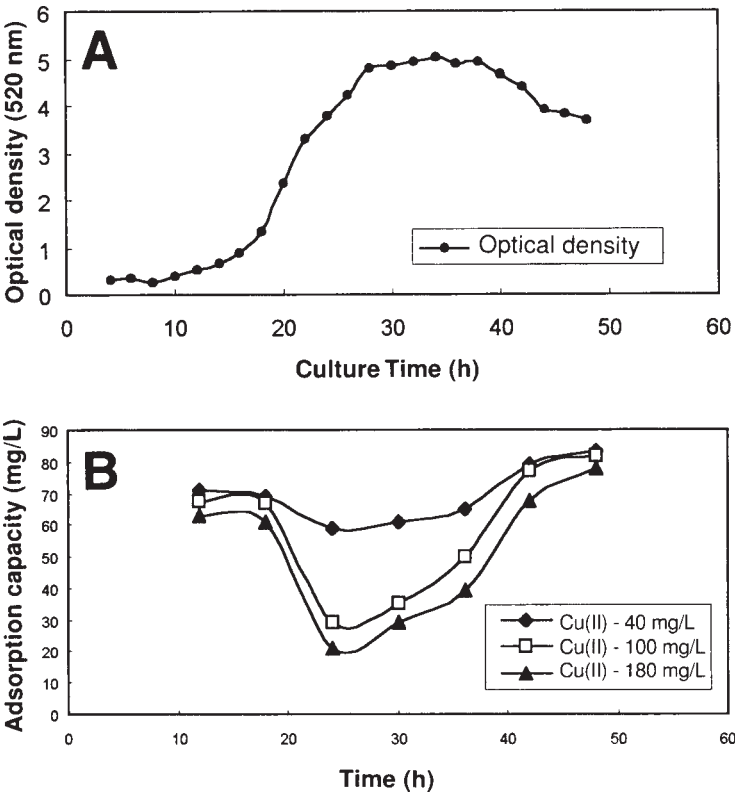

Fig. 3. **(A)** Growth curve of *P. putida 5-x* in SLM and **(B)** effect of growth phase and Cu^{2+} concentration on adsorption capacity.

plasma membrane degraded. However, there was no further degradation after the first adsorption-desorption cycle (Fig. 4). This implied that effective adsorption of Cu^{2+} by acid-pretreated cells is owing to the degradation of that plasma membrane, which may restrain the contact of Cu^{2+} with the activity center on the cell surface (such as protein or carboxylate groups). TEM also enables the comparison of typical cell surface appearance during various growth phases (14, 30, and 44 h). After copper uptake, early cells showed images that were slightly discerned because of the lack of contrast with the cell body (Fig. 5A). The latter cell of 30 hand 44 h exhibited a very visible electron-dense staining (Fig. 5B,C). Further X-ray analysis by the Department of Physics, Chinese University of Hong Kong, indicated that the electron-dense staining was copper. This was direct evidence that the adsorption capacity of cells in different growth phases to Cu^{2+} was different, and implied that the activity groups on the cell surfaces (such as protein or carboxylate groups) were less in the early cells and much more in the later cells.

The uptake of Cu(II) by *P. putida 5-x* was relatively fast (on the order of minutes). Ninety-five percent of the accumulated Cu(II) was obtained within the first 20 min of contact. The copper adsorption by *P. putida 5-x* was

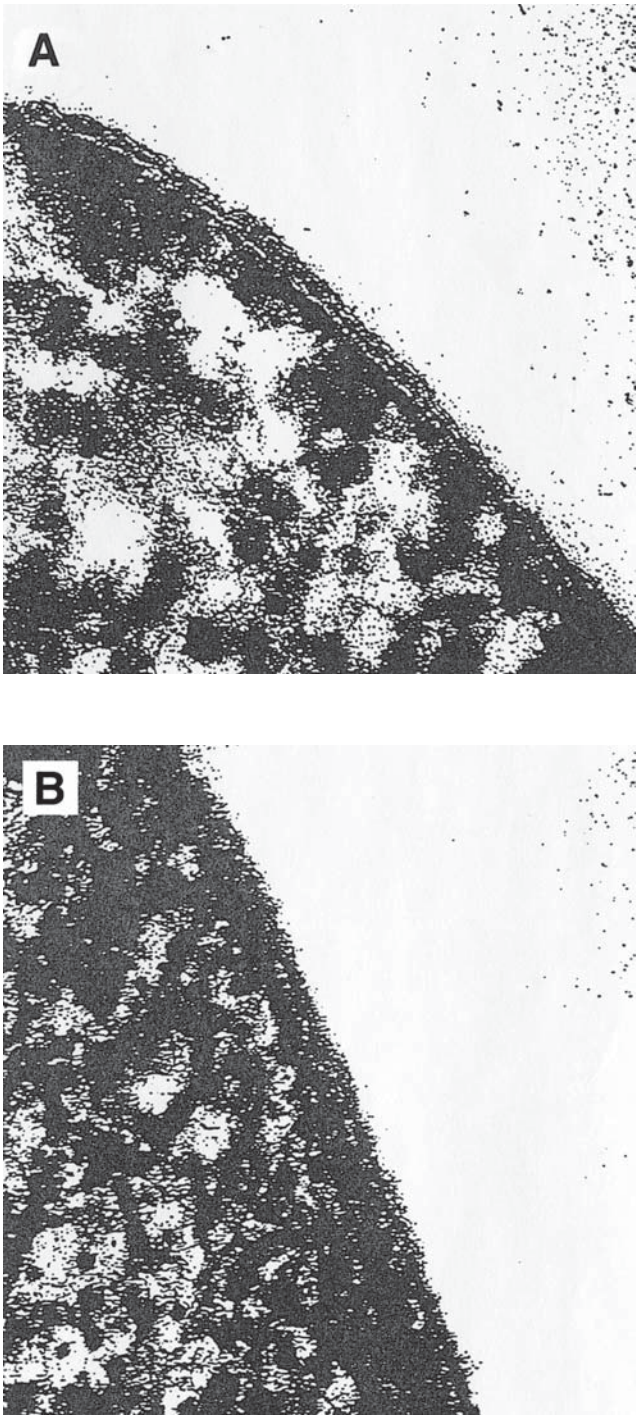

Fig. 4. Transmission electron micrographs of the cell walls and plasma membranes of magnetite-free cells of *P. putida 5-x*: **(A)** unpretreated cells; **(B)** cells after being pretreated with 0.6 *N* HCl; **(C)** first Cu(II) adsorption-desorption cycle. Magnification: ×100,000.

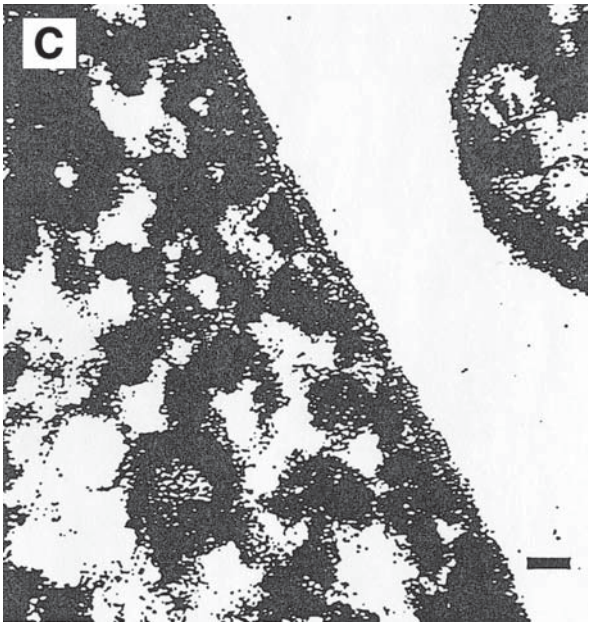

Fig. 4. *(continued)*.

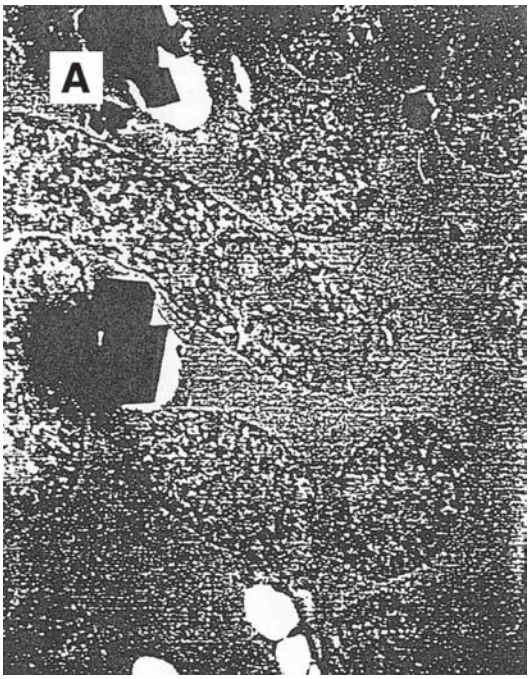

Fig. 5. Transmission electron micrographs of thin sections of *P. putida 5-x* in different growth phases after uptake of Cu^{2+}: **(A)** 14-h cell; **(B)** 30-h cell; **(C)** 44-h cell. Cells were exposed to Cu(II) (100 mg/L) in 10 m*M* MES buffer solution (pH 5.5). Magnification: ×100,000.

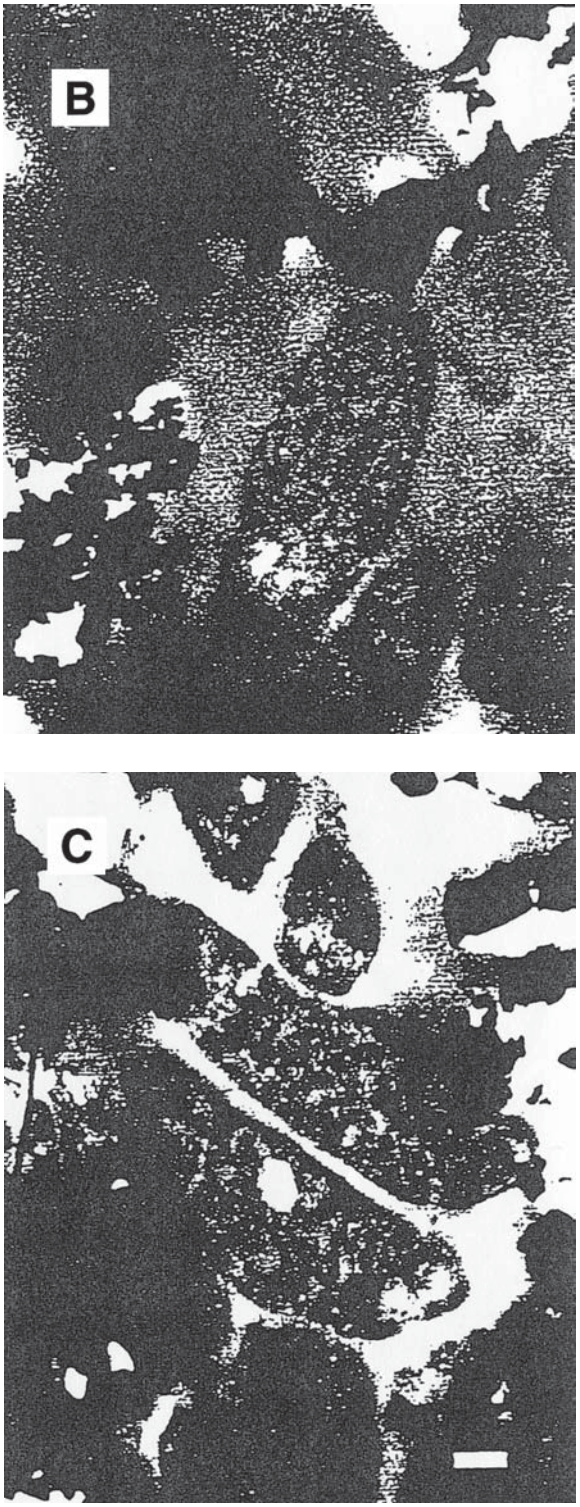

Fig. 5. *(continued)*.

reversible and took place mostly on the bacterial cell surfaces due to the presence of various polar groups in the structural components of cell walls, such as protein or carboxylate groups *(16,17)*. The first step relates the interaction of Cu(II) ions in a stoichiometric manner with accessible reactive groups on bacterial cell surfaces. During bacterial growth, these activity groups (such as proteins), which are not necessary for microorganism growth, were not produced in great numbers during fast cell cleavage (log growth phase), but only when cell cleavage reached the stationary phase (corresponding to the stationary growth and early death phases). These unnecessary proteins could then be produced in greater numbers. In this experiment, the variation of Cu^{2+} adsorption capacity of *P. putida 5-x* during various growth phases seemed to keep step with the production of these Cu^{2+}-dependent protein and carboxylate groups. TEM analysis provided evidence of a relationship between Cu^{2+}-dependent protein and Cu^{2+} adsorption capacity of *P. putida 5-x*.

In addition, a high Cu^{2+} concentration in aqueous solution was a disadvantage for the adsorption of *P. putida 5-x*, especially to the bacterial cells harvested during the early log growth phase. A high Cu^{2+} concentration in aqueous solution may lead Cu^{2+}-dependent protein to denaturation, and the spatial structure of the activity center of Cu^{2+}-dependent protein generated a variation of this disadvantage to Cu^{2+} adsorption. During the late stationary growth phase or early death phase, Cu^{2+}-dependent-protein was quite abundant on the cell surfaces, except some protein activity was inhibited by Cu^{2+}. The rest of the protein could still adsorb Cu^{2+} in aqueous solution, so the inhibition of Cu^{2+} to the adsorption ability of *P. putida 5-x* harvested during the late stationary growth phase or early death phase was not notable. By contrast, the effect of Cu^{2+} concentration on adsorption of *P. putida 5-x* harvested during the log growth phase was quite notable owing to the loss of Cu^{2+}-dependent protein during this period.

Conclusion

The magnetite-immobilized cells of *P. putida 5-x*, pretreated by dilute HCl, demonstrated better biosorption capacity than observed on fresh cells. The biosorption process was reversible. Bacterial cells harvested during the late stationary growth phase or early death phase had optimal adsorption capacity to Cu^{2+}, and resisted relatively high concentration Cu^{2+} in aqueous solution. The mechanism of the adsorption and acidic pretreatment on the cells was studied. TEM analyses indicated that acidic pretreatment degraded a plasma membrane on the cell surfaces to improve the adsorption capacity of cells to Cu^{2+}, and later cells adsorbed more Cu^{2+} on a single-cell surface than did early cells.

Acknowledgments

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