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ACCUMULATION OF NICKEL ION (Ni^{2+}) BY IMMOBILIZED CELLS OF *ENTEROBACTER* SPECIES

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SUMMARY: An *Enterobacter* species, isolated from electroplating effluent, could remove significant amount of nickel ion (Ni^{2+}) from growth medium and sewage effluent. In order to construct a bioreactor to remove Ni^{2+} from electroplating effluent, bacterial cells were immobilized in polyacrylamide beads. The highest removal capacity (RC, mg of Ni^{2+}/g of dry cells) and removal efficiency (RE, % of added Ni^{2+} removed by bacterial cells) of Ni^{2+} of immobilized bacterial cells were obtained by optimizing the growth conditions for the bacterial cells such as the composition of the growth medium, incubation time and incubation temperature; and the operational parameters of the bioreactor such as retention time and pH of the Ni^{2+} containing solution, respectively.

INTRODUCTION

Many industries such as electroplating and metal finishing discharge heavy metal-laden effluent into the environment. Toxic effects of heavy metals to various sensitive members of food chains have been well-documented (Pickering *et al.*, 1989, Reish *et al.*, 1989). Table 1 lists the mean concentrations of heavy metal ions in the effluent collected from a number of electroplating factories in Hong Kong. Among these heavy metal ion, nickel ion (Ni^{2+}) has the highest concentration in the electroplating effluent. There is no specific process in conventional sewage treatment to remove heavy metal ions, but large amounts of heavy metal ions accumulate in "activated sludge" - the active component of the biological sewage treatment. Thus low concentrations of heavy metal ions in raw sewage could cause significant reduction of the efficiency of biological sewage treatment by inhibiting microbes in activated sludge (Bagby and Sherrard, 1981). A number of physical and chemical methods have been developed to remove heavy metal ions from raw sewage. However these methods are industrially impractical either due to the high operational cost or the difficulty to treat the solid wastes subsequently generated (Tortora *et al.*, 1986). Recently, biological methods to remove heavy metal ions from raw sewage have drawn a widespread attention (Brierley *et al.*, 1986, Macaskie *et al.*, 1987). Microorganisms such as bacteria, yeasts and fungi (Nakajima and Sakaguchi, 1986), as well as algae (Khummongkol *et al.*, 1982) can accumulate large amounts of heavy metal ions. An *Enterobacter* sp., previously isolated from electroplating effluent, was shown to remove large amount of Ni^{2+} from culture medium and sewage effluent (Wong and Choi, 1988). Bacterial cells of the *Enterobacter* sp., grown in a minimal medium containing 10 mg/L of Ni^{2+}, removed about 34% of added Ni^{2+} from the medium. The Ni^{2+} RC of bacterial cells was about 8% of its dry weight (Wong and Choi, 1988). A number of other bacteria, yeasts and fungi have been shown to remove only an amount of Ni^{2+} about 0.6% of the dry weight of their biomass when grown in the medium containing about 3 mg/L of Ni^{2+} (Nakajima and Sakaguchi, 1986), while a strain of *Pseudomonas* sp. could remove an amount of Ni^{2+} about 1.2% the dry weight of the cells from the growth medium containing about 60 mg/L of Ni^{2+} (Bordons and Jofre, 1987). Kasan and Stegmann (1987) also reported a strain of *Enterobacter* sp. could remove only 20% of Ni^{2+} from the industrial cooling water with 100 mg/L of Ni^{2+}. Thus, the bacterium isolated by Wong and Choi (1988) seems to have greater Ni^{2+} removal ability than those microorganisms studied previously, and was proposed to be used to remove Ni^{2+} from industrial effluent. Due to their small size, it is difficult to recover these bacterial cells from industrial effluent after the removal of Ni^{2+}

Table 1. Mean concentrations of heavy metal ions in effluent collected from electroplating factories in Hong Kong[a].

Heavy Metal Ion	Concentration (mg/L)
Copper (Cu^{2+})	3.5
Chromium (Cr^{6+})	14.4
Nickel (Ni^{2+})	76.5
Zinc (Zn^{2+})	18.3

[a]Data provided by The Environmental Management Division, Hong Kong Productivity Council.

Immobilization of whole bacterial cells for removal of Ni^{2+} from industrial effluent has been proposed because of its large scale application. In addition, immobilized bacterial cells have better mechanical stability (Nakajima *et al.*, 1982). Since whole cell immobilization in a polyacrylamide gel provides a convenient laboratory model system, the effects of growth conditions for the bacterial cells and operational parameters of the bioreactor on the Ni^{2+} RC of the polyacrylamide-immobilized bacterial cells were determined.

MATERIALS AND METHODS

Culture media and solutions. The composition of Minimal Medium (MM) used in the present study was (in g/L): Trizma base, 6.05, NH_4Cl, 1.02, K_2HPO_4, 3.0, $FeSO_4.7H_2O$, 0.0003, $MgSO_4.7H_2O$, 0.5, $CaCl_2.2H_2O$, 0.03, NaCl, 1.0, and D-glucose, 4.0. The pH of the medium was adjusted to 7.0 with 1N HCl. Heavy metal ion stock solutions (10,000 mg/L of respective heavy metal ions) were prepared by dissolved either $CuCl_2.2H_2O$, 2.68 g; $NiCl_2.6H_2O$, 4.05 g; K_2CrO_4, 3.73 g; or $ZnCl_2.6H_2O$, 2.08 g in 100 mL double-distilled water and sterilized by autoclaving. Tris-buffer to resuspend bacterial cells and prepare Ni^{2+} containing solution was 10 mM Tris and the pH of the buffer was adjusted to 7.0 with 1 N HCl.

Growth of bacterial cells. Bacterial cells for immobilization were grown in MM at 30°C (or tested temperatures if stated) with shaking at 200 rpm. Growth of bacterial culture was followed colorimetrically at A_{520nm} by a Bausch & Lomb Spectronic 20D spectrophotometer. Bacterial cells were harvested at suitable time (usually after 48 hours incubation at 30°C) by centrifugation at 7,000 x g for 20 minutes at 4°C and then washed with Tris-buffer for immobilization or determination of the dry weight of bacterial cells (Wong and Choi, 1988).

Immobilization of bacterial cells. To prepare 8 % gel, 50 mg (dry weight pre-determined) of freshly harvested bacterial cells were suspended in 8 mL Tris-buffer and mixed with 2 mL of 40% polyacrylamide stock solution (36.4 g acrylamide and 3.4 g N,N'-methylene-bis-acrylamide dissolved in 50 mL Tris-buffer and then diluted with the same buffer to 100 mL) and 100 μL of ammonium persulfate (10%). After adding 10 μL N,N,N',N'-tetramethyl ethylenediamine (TEMED) into the mixture, polymerization was performed at 25°C for one hour. The gel was then crushed into small pieces (50-100 mesh), washed thoroughly with Tris-buffer, and suspended in minimal volume of Tris-buffer for the heavy metal ion uptake experiments.

Heavy metal ion uptake experiments. Batch type and continuous type heavy metal ion uptake experiments were performed by using immobilized bacterial cells. In the batch type heavy metal ion uptake experiment immobilized bacterial cells were suspended in 20 mL Tris-buffer with 100 mg/L of Ni^{2+} or other heavy metal ions, and packed into a column (1.5 cm x 15 cm). After immobilized

bacterial cells exposed to Ni^{2+} containing Tris-buffer for 60 minutes, the buffer was completely drained and the concentration of heavy metal ion in the buffer was determined by a Varian Spectra AA-10 atomic absorption spectrophotometer. For the continuous type heavy metal ion uptake experiment immobilized cells were packed into a column (1.5 cm x 15 cm), washed thoroughly with 100 mL Tris-buffer and then challenged with Tris-buffer containing various concentrations of Ni^{2+} or other heavy metal ions. Ten mL fractions were collected by using an ISCO Retriever IV fraction collector. The concentrations of heavy metal ion in these fractions were determined by the atomic absorption spectrophotometry. All experiments including controls were performed in duplicates. The removal capacity (RC) of immobilized bacterial cells was defined as mg of heavy metal ion/g of dry cells, while the removal efficiency (RE) was defined as % of added heavy metal ion removed by bacterial cells.

Optimization of Ni^{2+} RC of immobilized bacterial cells. Effects of growth conditions of the bacterial cells including availability of nitrogen, phosphate, or sulfate, concentration of glucose, incubation temperature and incubation time on Ni^{2+} RC of immobilized bacterial cells were determined by using batch type heavy metal ion uptake experiments. The concentration of nitrogen (NH_4Cl), phosphate (K_2HPO_4) or sulfate ($MgSO_4.7H_2O$) in MM was reduced to the growth-limited level according to that reported by Bolton and Dean (1972). Effect of concentration of glucose on Ni^{2+} RC of immobilized bacterial cells was determined by growing bacterial cells in phosphate-limiting MM containing 0.1, 0.2, 0.4, 0.8, or 1.6% D-glucose. Effects of various incubation temperatures (e.g. 25, 30 and 37°C) and incubation times (e.g. 24, 48 and 72 hours) on Ni^{2+} RC of immobilized bacterial cells were also determined. In order to study the effects of operational parameters of bioreactor such as retention time (i.e. the contact time) and pH of the Ni^{2+} containing buffer on Ni^{2+} RC of immobilized bacterial cells, continuous type heavy metal ion uptake experiments were performed with various flow rates (retention times of to 3, 10, 20 and 30 minutes) or Ni^{2+} containing buffer with different pH (e.g. 5, 6, 7, 8 and 9).

Effects of treatment of metabolic inhibitor and heat-killing on Ni^{2+} RC of immobilized bacterial cells. Metabolism-inhibited cells were prepared by suspending the cultured cells in Tris-buffer containing 1 mM sodium azide (NaN_3) for 1 hour. Then cells were washed twice with Tris-buffer. Heat-killed cells were prepared by suspending the cultured cells in Tris-buffer and heat treated in boiling water bath for 1 hour. Ni^{2+} RC of the azide- or heat-treated cells was then determined by the methods mentioned above.

Recovery of Ni^{2+} and regeneration of bioreactor. In the batch type heavy metal ion uptake experiment, after the pre-determined amount of Ni^{2+} in Tris-buffer was loaded and equilibrated at room temperature for 60 minutes, the buffer was completely drained. Then 20 mL of 0.5 M citrate buffer (pH=4.0) was added into the column and equilibrated at 25°C for another 10 minutes (Macaskie *et al.*, 1987). Finally, the acidic solution was completely drained from the column and the concentration of Ni^{2+} in the eluent was determined. The unloaded column was washed with 100 mL Tris-buffer before another heavy metal ion uptake experiment.

RESULTS AND DISCUSSION

Heavy metal ion RC of immobilized bacterial cells. Several immobilization matrice including alginic acid (calcium alginate), carrageenan and acyrlamide (all purchased from Sigma Chemical Company, St. Louis, Missouris) were tested for their metal ion removal capacities. Among them, the metal ion removal capacities of acrylamide (polyacrylamide gel) was insignificant (data not shown). Removal capacities of immobilized bacterial cells for Cu^{2+}, Cr^{6+} (as chromate anion, CrO_4^{2-}), Ni^{2+}, and Zn^{2+} were 4.5, 2.8, 8.8 and 1.8 mg of metal/g of dry cells, respectively. Among these four heavy metal ions, immobilized bacterial cells had the highest RC for Ni^{2+}, and thus subsequent experiments were focused on the optimization of removal of Ni^{2+} from the buffer by the immobilized bacterial cells. In addition, when 3.5 mg/L of Cu^{2+}, 14.4 mg/L of Cr^{6+} and 18.3 mg/L of Zn^{2+}, the mean concentrations of these heavy metal ions in the electroplating effluent (Table 1), was added solely or in bi- or tri-metallic combinations to Tris-buffer containing 100 mg/L of Ni^{2+},

only the presence of Zn^{2+} slightly reduced Ni^{2+} RC of immobilized bacterial cells (less than 10% of the control, data not shown). This result suggests that even the presence of high concentration of Zn^{2+} (18.3 mg/L) could only slightly affect the removal of Ni^{2+} by immobilized bacterial cells.

Optimization of growth conditions of the bacterial cells for Ni^{2+} removal. The importance of capsular polysaccharide in the removal of metal by *Klebsiella aerogenes*, a member of enteric bacteria similar to *Enterobacter* sp., was documented previously (Rudd *et al.*, 1983). In addition, the stimulating effect of nitrogen limitation (Duguid and Wilkinson, 1953) and inhibitory effect of phosphate limitation (Gahan *et al.*, 1967) on the production of capsular polysaccharide in *Klebsiella* (*Aerobacter*) sp. have been reported. The bacterial cells of the isolated *Enterobacter* sp. grown in MM with nutrient-limitation in nitrogen, phosphate or sulfate had higher Ni^{2+} RC (ranges from 12 to 25 mg of Ni^{2+}/g of dry cells) than that (RC=7 mg of Ni^{2+}/g of dry cells) of bacterial cells grown in MM without nutrient-limitation (Fig. 1). However, bacterial cells grown in the phosphate-limiting medium had the highest Ni^{2+} RC (Fig. 1). These results suggest that the capsular polysaccharide may not be the major bacterial component to remove Ni^{2+} from the medium because the production of capsular polysaccharide was inhibited by the limitation of phosphate (Gahan *et al.*, 1967). However, Duguid and Wilkinson (Duguid and Wilkinson, 1953) reported that the presence of excess sugar was required to enhance the production of capsular polysaccharide under the condition of nutrient-limitation. To study whether 0.4% glucose in MM provided excess sugar for bacterial cells to produce capsular polysaccharide, phosphate-limiting MM was supplemented with various concentrations of D-glucose to produce bacterial cells. Bacterial cells grown in the phosphate-limiting media with higher concentrations of D-glucose had the higher Ni^{2+} RC (Fig. 2). However, bacterial cells grown in the phosphate-limiting MM with 0.4% of D-glucose had Ni^{2+} RC about 80% of that of bacterial cells grown in the phosphate-limiting MM with 1.6% D-glucose. This result supports that the capsular polysaccharide of the bacterial cells involves but does not play the major role in the Ni^{2+} removal. In order to reduce the cost to produce bacterial cells as bio-sorbent, phosphate-limiting MM with 0.4% D-glucose was used for the following experiments. The incubation temperature and incubation time of bacterial culture also affected Ni^{2+} RC of the bacterial cells. Bacterial cells incubated at 30°C had the highest Ni^{2+} RC (Fig. 3) and 30°C was the optimal growth temperature for the bacterium. Also, bacterial cells from the 48-hour culture had the highest Ni^{2+} RC (Fig. 4). From the pre-determined growth curve, bacterial culture after 48 hours incubation was in the stationary phase of growth.

Optimization of operational parameters of bioreactor for the Ni^{2+} removal. Retention time between 10 to 30 minutes was sufficient for the maximum uptake of Ni^{2+} by immobilized bacterial cells (RC=26 mg of Ni^{2+}/g of dry cells, Fig. 5). Such a short retention time (i.e. high flow rate) ensures the bioreactor with immobilized bacterial cells can handle a large volume of influent within a short period of time. Maximum removal of Ni^{2+} by immobilized bacterial cells was obtained in the Ni^{2+} containing buffer with pH 7 to 8 (Fig. 6). This result suggests that this bioreactor can even be used to remove Ni^{2+} from the industrial effluent with neutral or slight alkaline pH.

Prospective. Since azide-treatment and heat-killing didn't significantly affect Ni^{2+} RC of the immoblized cells (data not shown), it suggested that Ni^{2+} uptake in these cells is metabolic-independent. There was about 80% of Ni^{2+} bound to immobilized bacterial cells could be recovered from the column by eluting with citrate buffer. In addition, Ni^{2+} RC of citrate-buffer treated immobilized bacterial cells was only about 80% of the untreated immobilized bacterial cells. The reduction of Ni^{2+} RC of immobilized bacterial cells in the regenerated bioreactor should be due to the pre-occupation of the 20% un-recovered Ni^{2+}. However, Ni^{2+} RC of the immobilized bacterial cells didn't change significantly in 5 continuous load-unload cycles. These results suggest that it is feasible to use the bioreactor for continuous operation to remove and recover Ni^{2+} from solution.

In order to determine the effect of concentration of Ni^{2+} in buffer on Ni^{2+} RE of the bioreactor with immobilized bacterial cells, bacterial cells grown in phosphate-limiting MM supplemented with 0.4% D-glucose for 48 hours at 30°C were immobilized and packed into glass column (1.5 x 15 cm). Continuous type heavy metal ion uptake experiment was performed in which buffer with 10, 50, 100 or

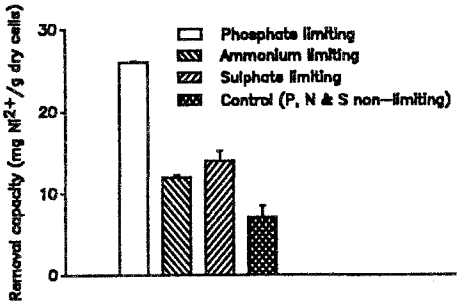

Figure 1. Ni^{2+} RC* of immobilized cells of *Enterobacter* sp. grown in various nutrient-limiting growth media. The basal medium is MM.

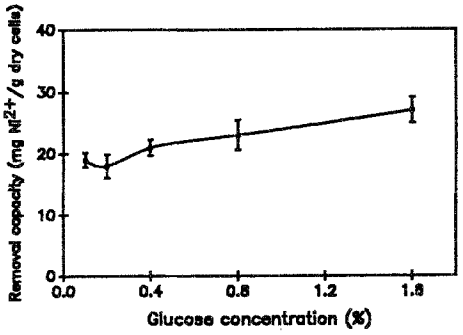

Figure 2. Effects of concentration of D-glucose on Ni^{2+} RC* of immobilized cells of *Enterobacter* sp..

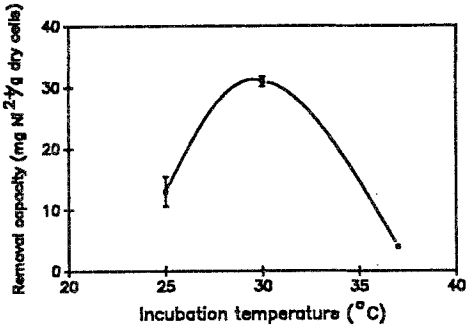

Figure 3. Effects of incubation temperature on Ni^{2+} RC* of immobilized cells of *Enterobacter* sp..

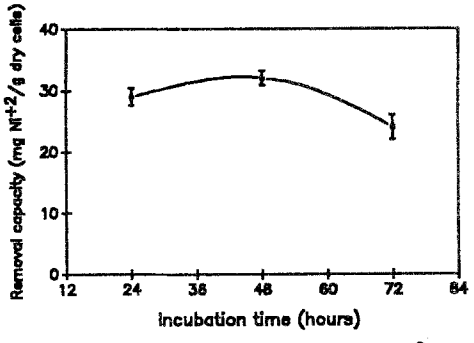

Figure 4. Effects of incubation time on Ni^{2+} RC* of immobilized cells of *Enterobacter* sp..

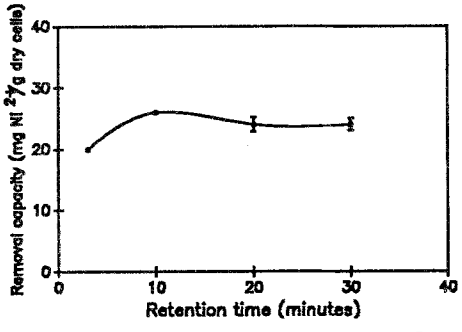

Figure 5. Effects of retention time on Ni^{2+} RC* of immobilized cells of *Enterobacter* sp..

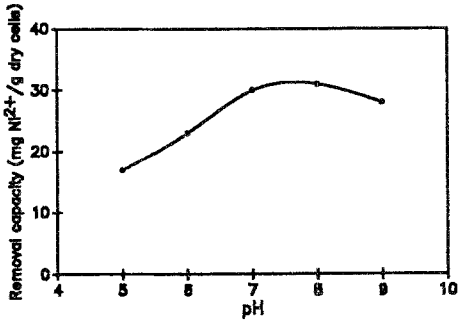

Figure 6. Effects of pH of Ni^{2+} containing buffer on Ni^{2+} RC* of immobilized cells of *Enterobacter* sp..

*RC=Removal capacity. Each point represents mean value±standard deviation of duplicates.

150 mg/L of Ni^{2+} was loaded into the bioreactor and Ni^{2+} RE of the bioreactor was determined. The bioreactor with immobilized bacterial cells could remove a amount of Ni^{2+} about 3% of their dry weight (i.e. RC=30 mg of Ni^{2+}/g of dry cells) but removed only 42, 40, 30 and 26% of added Ni^{2+} from Tris-buffer containing 10, 50, 100 and 150 mg/L of Ni^{2+}, respectively. In order to use this bioreactor to completely remove Ni^{2+} from industrial effluent, further experiments to study the possibility of increasing the Ni^{2+} RE of the bioreactor by using bigger column or link-columns, etc. will be performed.

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