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Simultaneous biodegradation of Ni–citrate complexes and removal of nickel from solutions by *Pseudomonas alcaliphila*

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**A B S T R A C T**

The objective of this study was to study the simultaneous biodegradation of Ni–citrate complexes and removal of Ni from solutions by *Pseudomonas alcaliphila*. Adding excess citrate to 1:1 Ni–citrate complexes promoted the degradation of the complexes and removal of Ni. The alkaline pH generated by the metabolism of excess citrate caused partial dissociation of citrate from the Ni–citrate complexes, allowing degradation, and the released Ni was removed through bioaccumulation and precipitation. Addition of Fe\(^{3+}\) enhanced the degradation of Ni–citrate complexes and removal of Ni from solutions. The displacement of Ni from recalcitrant Ni–citrate complexes by Fe\(^{3+}\) and subsequent biodegradation of the degradable Fe(III)–citrate complex resulted in complete metabolism of citrate. The almost complete removal of Ni (>98%) can be attributed to the combination of coprecipitation with Fe\(^{3+}\), bioaccumulation and precipitation. *P. alcaliphila* potentially could be applied in the treatment of effluent containing Ni–citrate complexes.

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1. Introduction

Complexing agents are widely used for metal finishing, painting, dyeing, photography and electroplating to enhance solubility of metals (Gyliene et al., 2009). They are also used in the extraction of toxic metals and radionuclides from wastes, sludges, sediments, and contaminated soils (Francis and Dodge, 1998). However, when treating metal-bearing wastewater, the presence of complexes makes chemical precipitation less effective (Malik, 2004), especially when the complexes are in excess of the metals (Gyliene et al., 2004). Adsorption, ion exchange and reverse osmosis processes have been utilized for the removal of chelated metals from solutions (Gyliene et al., 2009; Juang et al., 2006; Lu et al., 2010; Ozaki et al., 2002), but no satisfactory chemical or physical methods have been developed to cheaply remove or recover chelated metals from dilute solutions. One way by which metals can be released from complexes is biodegradation (Francis et al., 1992; Thomas et al., 1998).

Among the complexing agents, citrate is widely used in electroplating baths to avoid formation of insoluble metal compounds, and the wastes generated contain soluble metal–citrate complexes (Tarozaite et al., 2005). Citrate can be utilized as a carbon and energy source by a variety of microorganisms (Suzuki et al., 2005); however, the formation of metal-citrate complexes limits the utilization of citrate by bacteria (Bergsma and Konings, 1983). The inability of bacteria to metabolize certain metal-citrate complex is not due to the toxicity of the metals, but a lack of transport systems for metal–citrate complexes or the inability of the citrate-degrading enzymes to utilize the complexes as substrate or both (Joshi-Tope and Francis, 1995). The transport of metal–citrate complexes inside the cell is determined by the type of complex between metal and citrate (Francis et al., 1992). For example, Zn and Ca form mononuclear bidentate complexes and can be transported inside the cell, whereas Cd, Cu and Pb form mononuclear tridentate, and U forms binuclear complexes that cannot be transported inside the cell (Joshi-Tope and Francis, 1995). Metal-citrate complexes are metabolized intracellularly by aconitase and citrate lyase (Joshi-Tope and Francis, 1995). During the metabolism process, the toxicity of metals released from the complexes may be alleviated by a cation efflux system (Nies, 1999).

Nickel, a commonly used metal for electroplating, forms different types of stable complex with citrate. In the pH range 5–8, Ni and citrate are present predominantly as a mononuclear bidentate [Ni\(\text{Cit}\)]\(^{2-}\) complex (Hedwig et al., 1980). Above pH 8, the complex exists in a tridentate form involving the hydroxyl group of citrate, and above pH 9, it exists in a polymeric form [Ni\(_4\)OH\(\text{Cit}\)]\(^{8-}\) (Still and Wikberg, 1980; Strouse et al., 1977). The bidentate Ni–citrate complex (Tarozaite et al., 2005). Citrate can be utilized as a carbon and energy source by a variety of microorganisms (Suzuki et al., 2005); however, the formation of metal-citrate complexes limits the utilization of citrate by bacteria (Bergsma and Konings, 1983). The inability of bacteria to metabolize certain metal-citrate complex is not due to the toxicity of the metals, but a lack of transport systems for metal–citrate complexes or the inability of the citrate-degrading enzymes to utilize the complexes as substrate or both (Joshi-Tope and Francis, 1995). The transport of metal–citrate complexes inside the cell is determined by the type of complex between metal and citrate (Francis et al., 1992). For example, Zn and Ca form mononuclear bidentate complexes and can be transported inside the cell, whereas Cd, Cu and Pb form mononuclear tridentate, and U forms binuclear complexes that cannot be transported inside the cell (Joshi-Tope and Francis, 1995). Metal-citrate complexes are metabolized intracellularly by aconitase and citrate lyase (Joshi-Tope and Francis, 1995). During the metabolism process, the toxicity of metals released from the complexes may be alleviated by a cation efflux system (Nies, 1999).

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complex can be biodegraded by bacteria; however, the tridentate and polymeric Ni–citrate complexes are recalcitrant to biodegradation (Francis et al., 1996).

In a medium containing Ni and citrate in a ratio of 1:1, 95 and 100% of the citrate was utilized by Pseudomonas aeruginosa and P. putida, respectively, and the addition of inorganic phosphate as a precipitant promoted Ni removal effectively (Thomas et al., 2000). Francis et al. (1996) observed that 70% of the Ni–citrate complexes were degraded by P. fluorescens in medium with a 1:1 Ni:citrate content and buffered at pH 6.1, and that the addition of Fe3+ significantly promoted the degradation of Ni–citrate complex and removal of Ni from solution. Since previous studies focused on the biodegradation of Ni–citrate complexes in the media with equimolar concentrations of Ni and citrate, the present study investigated the biodegradation of Ni–citrate complexes and removal of nickel in media with excess citrate by Pseudomonas alcaliphila. The effect of Fe3+ on the biodegradation of Ni–citrate complexes and removal of Ni was also examined. The work will contribute information needed for treatment of wastewater by bioaugmentation.

2. Methods

2.1. Microorganism and growth conditions

P. alcaliphila, obtained from Chengdu Institute of Biology (Chengdu, China), was grown in modified Giltay medium before being used as inoculum. The modified Giltay medium contained the following components (per liter): 0.5 g of KNO3, 0.5 g of KH2PO4, 1.0 g of MgSO4·7H2O, 0.01 g of CaCl2·2H2O, 0.05 g of FeCl3·6H2O and 5 g of sodium citrate. Before transferring into 250-ml Erlenmeyer flasks containing 100 ml defined mineral salts medium, the culture was aerobically incubated at 30 °C on a rotary shaker at 120 rpm. The cells were harvested after about 18 h of cultivation, by centrifugation at 10,000g for 10 min at 20 °C. The pellets were washed with sterilized deionized water, twice, and re-suspended in defined mineral salts medium at a final concentration of 0.1 mg/l volatile suspended solids (VSS).

The defined mineral salts medium contained (per liter) 0.3 g of KNO3, 0.01 g of MgSO4·7H2O, 0.01 g of FeSO4·7H2O and 0.05 g of glycerol 2-phosphate. Glycerol 2-phosphate was used to provide phosphate for cell synthesis. NiCl2·6H2O was used as the source of Ni, and citrate was added in the form of sodium citrate. The amount of Ni and citrate added to the medium were calculated according to experiment demands. The culture was grown aerobically at 30 °C on a rotary shaker at 120 rpm. All media for research were sterilized through autoclaving (121 °C, 15 min) or syringe filtration (0.22 μm).

2.2. Biodegradation of Ni–citrate complexes

The degradation of Ni–citrate complexes by P. alcaliphila was investigated in medium containing 1 mM Ni and citrate. For comparison, the degradation of uncomplexed citrate was investigated in medium containing 1 mM citrate. The initial pH was adjusted to 7.0 with HCl or NaOH. Aliquots were taken periodically, filtered through a 0.22-μm filter, and analyzed for Ni and citrate.

2.3. Effect of excess citrate on degradation of Ni–citrate complexes and removal of Ni

Ni/citrate molar ratios of 1:2, 1:6 and 1:10 were studied. The initial Ni concentration was 1 mM. The initial pH was adjusted to 7.0 with HCl or NaOH. Aliquots were periodically removed to determine pH, Ni and citrate after 0.22-μm filtration. Another nine Erlenmeyer flasks were prepared at the same condition for each treatment, one of them was opened periodically and analyzed for biomass.

2.4. Effect of pH on degradation of Ni–citrate complexes and removal of Ni

The medium used to study the effect of pH on the degradation of Ni–citrate complexes contained 1 mM Ni and citrate. Barbitral sodium (1 g/l) was used to maintain the pH of medium at 7.3, 8.3 and 9.3. Aliquots were withdrawn periodically, filtered through a 0.22-μm filter, and analyzed for citrate and Ni.

2.5. Effect of Fe3+ on the degradation of Ni–citrate complexes and removal of Ni

Three experiments were conducted in the presence of Fe3+, added as FeCl3·6H2O. The treatments consisted of (i) 1:2 Ni:citrate plus 1 mM Fe3+, (ii) 1:6 Ni:citrate plus 1 mM Fe3+ and (iii) 1:10 Ni:citrate plus 1 mM Fe3+. The initial pH was adjusted to 7.0 with HCl or NaOH. Aliquots were withdrawn periodically, filtered through a 0.22-μm filter, and analyzed for citrate, pH, Ni and Fe.

2.6. Distribution and uptake of metals in P. alcaliphila after incubation

The distribution and uptake of metals was evaluated using the modified method of Perez-Rama and Alonso (2002). After 59 h of incubation, the 1:2, 1:6 and 1:10 Ni:citrate cultures, in triplicate, were centrifuged (13,000g for 10 min) and at 72 h, the 1:2, 1:1:2, 1:1:6 and 1:1:10 Ni:Fe:citrate cultures, in triplicate, were centrifuged (13,000g for 10 min). The supernatant was used to determine the residual metals in solution, and the pellets were used to analyze the cellular distribution of Ni and Fe.

The collected pellets were resuspended for 20 min in 40 ml of 20 mM EDTA at pH 6.5. The cells were centrifuged at 13,000g for 10 min and the supernatant was used to determine the metals released. The washed pellet was digested with concentrated HNO3 and HClO4 (100 °C, 20 min) for the determination of intracellular metals.

2.7. Scanning electron microscopy (SEM)

To prepare the SEM specimen, P. alcaliphila grown in the 1:1:10 Ni:Fe:citrate medium for 72 h and P. alcaliphila grown in Giltay medium for 20 h were collected via centrifugation (13,000g for 6 min). The harvested cells were washed twice with distilled water, layered onto cover slips, fixed in 3% glutaraldehyde buffered with 0.2 M sodium cacodylate at 4 °C overnight and washed with the same buffer twice. The samples were gradually dehydrated by incubating in 40, 50, 60, 70, 80, 90 and 100% ethanol and stored in 100% ethanol. Specimens in 100% ethanol were dried at CO2-critical point. The samples were sputter-coated with 15-nm Au particles, and viewed under a field emission scanning electron microscope (Hitachi Co., Japan S4800). Energy dispersive X-ray spectroscopy (EDS) of the selected areas within SEM sections was carried out using an Oxford EJ150 instrument coupled to the scanning electron microscope.

2.8. Analytical procedures

The concentrations of nickel were determined by atomic absorption spectrometry (AAS) using a Z2000 polarized Zeeman atomic absorption spectrophotometer (Hitachi, Japan). Citrate concentrations were analyzed on a C18 column (Welch Materials, Inc.) using high-performance liquid chromatography (Shimadzu) with a UV–vis detector at 210 nm. Aqueous culture samples were analyzed using a mobile phase of methane and 7.5 mM H3PO4 mixed
3. Results and discussion

3.1. Biodegradation of Ni–citrate complexes

Citrate was degraded completely in 24 h at a rate of 41.67 μmol/h, and the final pH of the medium increased to 8.83 (Fig. 1). Ni–citrate complexes were degraded after a lag of 9 h (Fig. 1). The bacteria degraded only 15% of the Ni–citrate complexes, with a concurrent increase in pH from 7.0 to 7.67, and all nickel was detected in the solution throughout the experiment.

In 1:1 Ni:citrate medium, 99.9% of the citrate was in the Ni–citrate complex form at neutral pH (Bergsma and Konings, 1983), and since Ni was not toxic to bacteria when complexed with citrate (Francis et al., 1996), the lag phase observed was likely due to the time needed for the induction of the transport factors for Ni–citrate complexes (Joshi-Tope and Francis, 1995). The relatively low level of degradation of Ni–citrate may have been due to toxicity of released nickel ion (Francis et al., 1996).

3.2. Biodegradation of Ni–citrate complexes and removal of Ni in media with excess citrate

In 1:2 Ni:citrate medium, 1.23 mM of citrate was degraded at a rate of 23.15 μmol/h, 0.77 mM citrate and 0.87 mM Ni remained in solution at the end of the experiment (Fig. 2a). In contrast, 5.4 mM of the citrate was degraded at a rate of 101.89 μmol/h in the 1:6 Ni:citrate medium, with 0.6 mM citrate and 0.72 mM Ni remaining in solution at the end of the inoculation (Fig. 2b). In the 1:10 Ni:citrate medium, 9.49 mM of the citrate was degraded at the rate of 179.09 μmol/h, with 0.51 mM residual citrate and 0.65 mM residual Ni in solution at the end of the experiment (Fig. 2c). Compared to the culture with equimolar concentrations of Ni and citrate, a much shorter lag period (~2 h) in the media with excess citrate was observed. These results indicated that the presence of excess citrate promoted the degradation Ni–citrate complexes and removal of Ni from solution.

Biomass increased rapidly to 0.04, 0.26 and 0.38 g/l without a lag period in the 1:2, 1:6 and 1:10 Ni:citrate media within 29 h, respectively (Fig. 2a–c). The pH of the 1:2, 1:6 and 1:10 Ni:citrate media increased gradually from 7.0 to 8.52, 9.42 and 9.58, respectively (Fig. 2a–c). The pH increase may have been caused by simultaneous transport and metabolism of proton with citrate and Ni–citrate complexes into the cell (Bergsma and Konings, 1983; Willecke et al., 1973). The higher the availability of citrate in the media, the higher the pH and biomass productivity.

In the 1:2, 1:6 and 1:10 Ni:citrate media, the pH reached 8.3 and biomass reached a plateau at 35 h; however, due to the presence of residual citrate, Ni could remain in solution and was not removed. When the citrate level fell below 1 mM and was not sufficient to chelate all of the Ni in solution, removal of Ni commenced. At the end of the incubation, 13, 28 and 35% of the Ni was removed from the 1:2, 1:6 and 1:10 Ni:citrate medium, respectively. Table 1 shows the distribution and uptake of Ni in P. alcaliphila grown in the 1:2, 1:6 and 1:10 Ni:citrate medium. Less than 1% of the removed Ni was found inside the bacteria, which indicated that removal of Ni were mostly through precipitation at alkaline pH and biosorption on the surface of bacteria.

During metabolism of citrate, the Ni to citrate ratio reached about 1 at 48 and 29 h in the 1:2 and 1:6 Ni:citrate media, respectively (Fig. 3), and the pH increased to between 8 and 9 (Fig. 2a and b), resulting in the Ni–citrate complexes to be present in tridentate form. In the 1:6 and 1:10 Ni:citrate media, when the Ni:citrate ratio increased to be about 1.3 at 35 h (Fig. 3) and pH increased to above 9 (Fig. 2b and c), the Ni–citrate complex was present in polymeric form. According to previous studies, Ni–citrate complexes in tridentate and polymeric form were recalcitrant to biodegradation (Francis et al., 1992; Francis et al., 1996). However, metabolism of citrate from tridentate and polymeric Ni–citrate complexes was observed in the 1:2, 1:6 and 1:10 Ni:citrate media, which could be explained by the possible ability of P. alcaliphila to degrade Ni–citrate complexes in tridentate and polymeric form, or by the pH increase causing the dissociation of citrate from the Ni–citrate complex for degradation.

3.3. Effect of pH on citrate degradation and Ni removal

In medium buffered at pH 7.3, metabolism of citrate was observed after a 9-h lag at the rate of 9.33 μmol/h, with 17% citrate degradation (Fig. 4a). All nickel was detected throughout the experiment (Fig. 4b). The citrate metabolism characteristics in 1:1 Ni:citrate medium buffered at pH 7.3 was similar to that in unbuffered 1:1 Ni:citrate medium. This indicated that the presence of barbital sodium as buffer did not cause the dissociation of citrate from Ni–citrate complexes.

In 1:1 Ni:citrate media buffered at pH 8.3 and 9.3, 24 and 54% of the citrate was degraded at the rate of 26.67 and 60 μmol/h, respectively (Fig. 4a). With the degradation of citrate, 17 and 40% of the Ni was removed from the 1:1 Ni:citrate medium buffered at pH 8.3 and 9.3, respectively (Fig. 4b), and bluish-green precipitate was observed. The lag period was much shorter in medium buffered at alkaline pH, and the citrate degradation rate at alkaline pH was much faster than that at neutral pH. After the rapid decrease of citrate during the initial phase, citrate concentration remained constants to the end of the experiments in the 1:1 Ni:citrate media buffered at pH 8.3 and 9.3. These results indicated that biodegradation of recalcitrant complexes did not occur and that the citrate utilized was free citrate.

At alkaline pH, Ni may form soluble nickel hydroxyl complexes, and citrate was dissociated from the Ni–citrate complexes. Although the tridentate and polymeric Ni–citrate complex themselves were nonbiodegradable, the dissociation of citrate from the Ni–citrate complexes at alkaline pH raised the bioavailability of citrate (Huang et al., 1998). In medium buffered at pH 9.3, more citrate was dissociated from the Ni–citrate complexes, so the citrate degradation percentage in medium buffered at pH 9.3 was higher than that in medium buffered at pH 8.3. After the rapid...
degradation of free citrate dissociated from the Ni–citrate complexes, a considerable amount of citrate and Ni remained as tridentate and polymeric Ni–citrate complexes, and no further metabolism of citrate was observed in media buffered at alkaline pH. Due to the low level of citrate bioavailability (less than 1 mM) in the buffered media, no significant growth was observed throughout the experiments. Therefore, Ni removal in media buffered at pH 8.3 and 9.3 can be attributed to precipitation, which also reduced the toxicity of Ni.

These results indicated that the pH increase attained from the metabolism of excess citrate enhanced the degradation of Ni–citrate complexes and removal of Ni in the 1:2, 1:6 and 1:10 Ni:citrate media. However, a significant amount of citrate and Ni remained in solution as recalcitrant Ni–citrate complexes to the end of the experiments.

Fig. 2. The variation of Ni, biomass, citrate and pH in the 1:2 (a), 1:6 (b) and 1:10 (c) Ni:citrate media after the inoculation of P. alcaliphila.
experiments. To further enhance the degradation of Ni–citrate complexes and removal of Ni, the effect of Fe$^{3+}$ was studied.

### 3.4. Effect of Fe$^{3+}$ on the biodegradation of Ni–citrate complexes and removal of Ni

In 1:1:2 Ni:Fe:citrate medium, citrate was degraded after a 48-h lag period, then citrate was degraded completely within 18 h (Fig. 5a). In the 1:1:6 and 1:1:10 Ni:Fe:citrate media, citrate was completely degraded within 54 and 44 h, respectively (Fig. 5b and c). With the complete degradation of citrate, Ni was also removed completely from solution. Compared to that in the 1:1:2 Ni:Fe:citrate medium, faster citrate utilization and Ni removal were achieved in the 1:1:6 and 1:1:10 Ni:Fe:citrate media. These results indicated that addition of 1 mM Fe$^{3+}$ to the 1:2, 1:6 and 1:10 Ni:Fe:citrate mediums facilitated the complete degradation of Ni–citrate complexes and removal of Ni, and the presence of excess citrate facilitated shortening the citrate metabolism and Ni removal times.

Compared to citrate degradation in the 1:2 Ni:citrate medium, a much longer lag period was observed in the 1:1:2 Ni:Fe:citrate medium, Fe$^{3+}$ and citrate are present as a 1:1 Fe(III)–citrate complex (Francis et al., 1992), therefore, all citrate was predominantly present in metal-citrate complex form in the 1:1:2 Ni:Fe:citrate medium. The lag period before citrate degradation may be due to the time needed for induction of the transport factor for uptake of Ni- and Fe(III)–citrate complexes. In contrast, due to the presence of excess citrate in the 1:1:6 and 1:1:10 Ni:Fe:citrate media, citrate was degraded without a lag phase (Fig. 5b and c).

In the 1:1:2, 1:1:6 and 1:1:10 Ni:Fe:citrate media, when the citrate level was above or equal to 2 mM, most of the Ni and Fe were chelated by citrate. Therefore, removal of Fe and Ni from solution was not observed in the initial stage of the experiments. It was not until the citrate level fell below 2 mM that the removal of Ni and Fe commenced in the three treatments. Removal of Ni and Fe presented a two-step process, a slow initial removal was followed by rapid removal, and removal of Ni commenced before that of Fe.

In the first step, citrate was completely metabolized and approximate 12, 32 and 37% of the Ni was removed from the 1:1:2, 1:1:6 and 1:1:10 Ni:Fe:citrate media, respectively. The removal of Fe commenced after that of Ni in the first step, and about 8 and 15% of the Fe was removed from the 1:1:6 and 1:1:10 Ni:Fe:citrate media, respectively.

The stability and biodegradability of Ni- and Fe(III)–citrate complexes may be responsible for these removal characteristics in the first metal removal step. The greater the value of the formation constant, the higher the affinity of the metal ion for citrate (Hughes and Poole, 1991). The formation constant of tridentate Fe(III)–citrate complex was relatively greater than that of all other metal-citrate complexes (Table 2), and tridentate Fe(III)–citrate complexes can be converted to the bidentate Fe(III)–citrate complexes for complete biodegradation (Francis and Dodge, 1993). When the citrate level decreased to between 1 and 2 mM, the citrate concentration was no longer sufficient to chelate all of the metals, and Ni(II) could

<table>
<thead>
<tr>
<th>Ni:citrate media</th>
<th>1:2</th>
<th>1:6</th>
<th>1:10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni (µmol)</td>
<td>13 ± 0.8</td>
<td>28 ± 1.2</td>
<td>35 ± 2.4</td>
</tr>
<tr>
<td>Residual Ni in media</td>
<td>87 ± 4.1</td>
<td>71.4 ± 3.1</td>
<td>65 ± 2.9</td>
</tr>
<tr>
<td>Bioremoved Ni Intracellular</td>
<td>0.8 ± 0.13</td>
<td>1.4 ± 0.12</td>
<td>1.2 ± 0.31</td>
</tr>
<tr>
<td>Extracellular</td>
<td>11.4 ± 1.8</td>
<td>27.2 ± 2.1</td>
<td>34.2 ± 1.2</td>
</tr>
</tbody>
</table>
not win the competition with Fe(III) for citrate (Hughes and Poole, 1991). As a result, all Fe remained as tridentate Fe(III)–citrate complexes in solution, and Ni was partially released from the Ni–citrate complex and removed through bioaccumulation and precipitation. Due to the metabolism of citrate, the pH increased to above 8 in the 1:1:2, 1:1:6 and 1:1:10 Ni:Fe:citrate media. The alkaline pH facilitated the dissociation of citrate from metal–citrate complexes for metabolism and removal of Ni from solution, whereas, a significant amount of Ni and Fe remained in solution in recalcitrant Ni- and Fe(III)–citrate complexes. Although the tridentate Fe(III)–citrate complexes themselves were recalcitrant to biodegradation, they can be converted to bidentate Fe(III)–citrate complexes for degradation (Francis and Dodge, 1993). After conversion and degradation of Fe(III)–citrate complexes, the released Fe(III) displaced Ni(II)
from recalcitrant Ni–citrate complexes for further degradation. Therefore, after the cycle of conversion, degradation and displacement, the metabolism of citrate by bacteria did not stop until the complete degradation of citrate in the 1:1:2, 1:1:6 and 1:1:10 Ni:Fe:citrate media was achieved. When the citrate level decreased to between 0 and 1 mM, Ni was further released and removed via bioaccumulation and precipitation, the removal of Fe also commenced. Maybe due to the stability of Fe(III)–citrate complexes, although the citrate concentration was quite low (<0.1 mM) and was no longer sufficient to chelate all of the Fe(III), only a small amount Fe was removed from solution before complete degradation of citrate during the first metal removal step.

In the second metal removal step, a significant amount of Ni and Fe were removed rapidly from solution after complete degradation.

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**Table 2**

The formation constants of metal-citrate complexes.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Formula</th>
<th>Type of complex</th>
<th>Formation constant (log K)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe³⁺</td>
<td>[Fe(OH)₂–citrate]²⁻</td>
<td>Bidentate</td>
<td>1.9–2.6</td>
<td>(Francis et al. (1992))</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>[Fe(OH)–citrate]⁻</td>
<td>Tridentate</td>
<td>9.4</td>
<td>(Francis et al. (1992))</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>[Ni–citrate]⁻</td>
<td>Bidentate</td>
<td>5.4</td>
<td>(Francis et al. (1992))</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>[Ni₄(OH)–citrate₃]⁵⁻</td>
<td>Polymeric</td>
<td>Unknown</td>
<td>–</td>
</tr>
</tbody>
</table>

---

**Table 3**

Distribution and uptake of Ni and Fe in *P. alcaliphila* grown in the 1:1:2, 1:1:6 and 1:1:10 Ni:Fe:citrate media (mean and standard deviation of three replicates are shown).

<table>
<thead>
<tr>
<th>Ni:Fe:citrate media</th>
<th>1:1:2</th>
<th>1:1:6</th>
<th>1:1:10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni (µmol)</td>
<td>Fe (µmol) Ni (µmol) Fe (µmol) Ni (µmol) Fe (µmol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture broth</td>
<td>Bioremoved metals</td>
<td>98.5 ± 2.1</td>
<td>98.5 ± 3.1</td>
</tr>
<tr>
<td>Residual metals in media</td>
<td>1.4 ± 0.16</td>
<td>1.5 ± 0.12</td>
<td>1.2 ± 0.15</td>
</tr>
<tr>
<td>Bioremoved metals</td>
<td>Intracellular</td>
<td>1.2 ± 0.11</td>
<td>0.9 ± 0.11</td>
</tr>
<tr>
<td>Extracellular</td>
<td>97.5 ± 3.2</td>
<td>97.8 ± 2.4</td>
<td>97.2 ± 4.1</td>
</tr>
</tbody>
</table>

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**Fig. 6.** SEM images and EDS analysis: (a) *P. alcaliphila* grown in the Giltay medium; (b) *P. alcaliphila* grown in the 1:1:10 Ni:Fe:citrate medium; (c) EDS analysis of the particles adhering to the surface of *P. alcaliphila* (point A indicated by the arrow); (d) EDS analysis of the particles scattered around *P. alcaliphila* (point B indicated by the arrow).
of citrate in the 1:1:2, 1:1:6 and 1:1:10 Ni:Fe:citrate media. In the absence of citrate, precipitation of Fe(III) begins at pH values 2–3, and precipitation of Ni begins at pH higher than 8 [Hughes and Poole, 1991]. In the three treatments, after complete metabolism of citrate, the simultaneous removal of Fe and Ni may be due to co-precipitation (Francis et al., 1996).

At the end of the incubation, Ni and Fe were almost completely removed from the 1:1:2, 1:1:6 and 1:1:10 Ni:Fe:citrate media. Less than 1.5% of the removed Ni and Fe was found inside the bacteria grown in the 1:1:2, 1:1:6 and 1:1:10 Ni:Fe:citrate media (Table 3). This finding indicated that removal of metals occurred mostly extracellularly. Compared with the smooth surface of bacteria grown in the Giltay medium (Fig. 6a), the surface of bacteria from 1:1:10 Ni:Fe:citrate medium was much rougher (Fig. 6b). SEM also showed particles scattered around the cells (Fig. 6b). EDS analysis indicated that the particles adhering to the surface of cells and the particles scattered around the cells all contained Fe and Ni (Fig. 6c and d). These particles may be composed of ferric hydroxide containing Ni formed during the process of co-precipitation.

Francis et al. (1996) thought that Ni co-precipitation with iron was responsible for the complete removal of Ni from 1:1:2 Ni:Fe:citrate medium. However, in the present study, the two-step metal removal process in the 1:1:2 Ni:Fe:citrate medium showed that the almost complete removal of Ni (>98%) from media was based on the combination of co-precipitation, bioaccumulation and precipitation. And with the increase in excess citrate in the media, more Ni was removed through bioaccumulation and precipitation.

During citrate metabolism and Ni removal, precipitation and co-precipitation alleviated the toxicity of Ni. The cation efflux system in the cell membrane of *P. alcaliphila* may also be responsible for detoxification of metal released from the complexes (Nies, 1999).

4. Conclusions

In 1:1 Ni-citrate medium, *P. alcaliphila* degraded only 15% of the Ni-citrate complexes. In the media with excess citrate, the pH increase promoted the bioavailability of citrate from recalcitrant Ni-citrate complexes and enhanced the removal of Ni from solution. Addition of Fe³⁺ caused the complete metabolism of citrate, and resulted in almost complete removal of Ni (>98%). *P. alcaliphila* potentially could be applied to biodegrade Ni–citrate complexes and remove Ni from wastewater under aerobic conditions.

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References


