Cobalt(II) bioaccumulation and distribution in *Rhodopseudomonas palustris*

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**ABSTRACT**

Bioaccumulation by growing cells is a potential technique for the removal of heavy metals from wastewater. Cobalt bioaccumulation by *Rhodopseudomonas palustris* via different metabolic pathways was investigated for various pH levels, temperatures, co-cations and initial Co²⁺ concentrations. The distribution of cobalt and its chemical nature in *R. palustris* were examined by cell compartmentalization and X-ray diffraction analysis. Our results indicated that biomass accumulation efficiency and bioaccumulation capacity were, respectively, 84.9% and 287.18 mg/g for cultures with an initial Co²⁺ concentration of 160 mg/L. A pseudo-second-order kinetic model fits the removal process well (R² = 0.9508–0.9913). Co²⁺ removal efficiency was not significantly influenced by Cu²⁺, Zn²⁺, Cd²⁺, Mn²⁺ or Fe²⁺ but was influenced by Ni²⁺. Cobalt was distributed between the cell surface, periplasmic space, membrane and cytoplasm. The periplasmic space and membrane accounted for 57.37% and 27.95% of Co²⁺ uptake, respectively. X-ray diffraction analysis showed that cobalt ions were predominantly deposited in the cell as cobalt phosphate octahydrate. These results demonstrate that *R. palustris* could be used to remove cobalt from industrial wastewater; it would therefore be beneficial to further study the mechanism of cobalt bioaccumulation in this organism.

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**Introduction**

Pollution from heavy metals poses a significant threat to the environment and to human health due to the toxicity, persistence and non-biodegradability of various compounds that form the pollutants. Cobalt is an essential trace element and plays an important role in animals, yeasts, bacteria and plants [1]. However, excess cobalt is often discharged by industrial activities such as mining, metallurgy, electroplating, painting, pigmenting and lithography [2,3]. Such discharges can result in serious health effects for animals and humans, including contact dermatitis, paralysis, lung irritation and bone defects. Exposure to ionizing radiation is associated with an increased risk of developing cancer [4–7]. The permissible limits of cobalt in irrigation water and livestock wastewater are 0.05 and 1 mg/L, respectively (Environmental Bureau of Investigation, Canadian Water Quality Guidelines) [8]. A variety of technologies have been developed over the years to remove toxic cobalt from water, including chemical precipitation, ion exchange [9], reverse osmosis [10] and activated carbon adsorption [11]. However, most of these technologies are expensive, inefficient, labour-intensive or unselective.

Therefore, there is a great need for an alternative cobalt removal technology that is efficient and economical. Bioaccumulation by growing cells is a potential method for the removal of heavy metal from the environment [12,13].

In micro-organisms, the bioaccumulation of heavy metals arises from metabolism-independent extracellular adsorption (surface interaction of heavy metal ions with cell-surface functional groups) and metabolism-dependent uptake followed by intracellular accumulation. Intracellular accumulation is a more complex process and may involve the localization of metal ions within specific organelles, enzymatic detoxification and efflux pumps [14]. In parallel, growing cells can cleave organo-metallic complexes, degrade organic contaminants such as dyes [15] and 2,4-dichlorophenoxyacetic acid [16] and take up other inorganic ions such as ammonium, nitrate and phosphate [17]. *Rhodopseudomonas palustris* (*R. palustris*) is a purple photosynthetic bacterium and is widely found in soil, wastewater and activated sludge. *R. palustris* has extraordinary metabolic versatility, grows with or without oxygen and uses many alternative carbon and energy sources [18]. Most importantly, many people have demonstrated that *R. palustris* exhibits high tolerances and
enormous capacities for various metals [19,20]. The objective of this study is to investigate the effects of metabolic pathways, pH, temperature, co-cations and initial Co\textsuperscript{2+} concentrations on cobalt bioaccumulation in \textit{R. palustris}. To understand the distribution of cobalt within \textit{R. palustris} cells, cell compartmentalization and X-ray diffraction analysis were performed.

**Materials and methods**

**Strains and growth conditions**

\textit{R. palustris} was from our laboratory and was cultured in enrichment medium: 4 g DL-malic acid, 1 g (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 0.12 g MgSO\textsubscript{4}.7H\textsubscript{2}O, 0.6 g K\textsubscript{2}HPO\textsubscript{4}, 0.9 g KH\textsubscript{2}PO\textsubscript{4}, 0.02 g sodium ethylenediaminetetraacetic acid (EDTA), 75 mg CaCl\textsubscript{2}.2H\textsubscript{2}O, 1 mg vitamin B1, 1 mg nicotinic acid, 15 mg biotin and 1 mL trace element solution dissolved in 1 L distilled water [21]. The pH of the medium was adjusted to 6.5 with NaOH solution before autoclaving. \textit{R. palustris} was incubated anaerobically at 30°C under 2000 lx illumination for 60 h.

**Effect of metabolic pathways on cobalt bioaccumulation**

A 4 mL volume of growing cells in exponential phase (0.7–0.9 g dry cell/L) was suspended in 100 mL growth medium containing 80 mg/L Co\textsuperscript{2+}, which was prepared by dissolving Co(NO\textsubscript{3})\textsubscript{2}.6H\textsubscript{2}O in ultrapure water. \textit{R. palustris} was cultured anaerobically in the light and in the dark. Other cells were cultured aerobically in illuminated and dark orbital shakers (Huamei Biochemical Instrument, QHZ-98B, China) at 120 rpm. The optical densities of bacterial cultures were determined at 600 nm using a UV/Vis spectrophotometer. OD\textsubscript{600} measurements were then converted to corresponding dry cell weights. Residual Co\textsuperscript{2+} concentrations in supernatants were measured using an atomic absorption spectrophotometer (AAS, PerkinElmer AAnalyst 800, USA) following centrifugation (10,000 g, 10 min) and filtering through 0.22 \textmu m cellulose acetate membranes. Co\textsuperscript{2+} removal efficiencies were calculated using the following equation:

\[
\text{Removal efficiency(\%)} = \frac{(C_0 - C_e)}{C_0} \times 100 \quad (1)
\]

where \(C_0\) and \(C_e\) are the initial and equilibrium metal concentration (mg/L), respectively.

**Effect of pH and temperature on cobalt bioaccumulation**

Cobalt bioaccumulation was investigated by adding \textit{R. palustris} to medium containing 80 mg/L Co\textsuperscript{2+} and adjusted to various pH values (5.5–7.5) using NaOH solution. The effect of temperature on cobalt bioaccumulation was studied at pH 6.5 as noted earlier; the culture temperature varied between 20 and 40 °C.

**Effect of co-cations on cobalt bioaccumulation**

\textit{R. palustris} was incubated within medium containing 80 mg/L Co\textsuperscript{2+} and equivalent concentrations of Ni\textsuperscript{2+}, Fe\textsuperscript{2+}, Cu\textsuperscript{2+}, Zn\textsuperscript{2+}, Cd\textsuperscript{2+} or Mn\textsuperscript{2+}. Culture conditions were set to the optimal pH and temperature determined previously.

**Effect of initial cobalt concentration on cobalt bioaccumulation**

To assess the effect of Co\textsuperscript{2+} concentration on cell growth and metal uptake, experiments were performed with initial Co\textsuperscript{2+} concentrations ranging from 20 to 160 mg/L. Culture conditions were set to the optimal pH and temperature determined previously. A 2 mL volume of culture solution was withdrawn every 5 h. We monitored the dry cell weights and Co\textsuperscript{2+} concentrations in samples. After 80 h of cultivation, \textit{R. palustris} was harvested via centrifugation and dried at 60 °C for 4 h to determine the bioaccumulation capacity of the organism. The Co\textsuperscript{2+} bioaccumulation capacity was calculated using the following equation:

\[
q_e = \frac{(C_0 - C_e)V}{X} \quad (2)
\]

where \(q_e\) is the equilibrium Co\textsuperscript{2+} concentration in \textit{R. palustris} (mg/g), \(X\) is the biomass of \textit{R. palustris} (g dry cell/L) and \(C_0\) and \(C_e\) are the initial and equilibrium Co\textsuperscript{2+} concentrations (mg/L), respectively.

**Cell compartmentalization**

To determine the proportion of cobalt associated with surface exchange sites, the periplasmic space, the membrane fraction and the cytoplasm, a series of extractions were performed according to the modified method described by Kumar [22]. Cells grown with 80 mg/LCo\textsuperscript{2+} were harvested at stationary phase, then washed twice with 0.03 mol/L Tris-buffer (pH 8.0) containing 2.5 mmol/L EDTA. Metals associated with exchange sites on the cell surface were extracted by re-suspending the cell pellet in 5 mmol/L Ca\textsuperscript{2+} as Ca(NO\textsubscript{3})\textsubscript{2} and incubating for 30 min with gentle shaking at 80 rpm [23,24]. The suspension was centrifuged at 10,000 g for 15 min, and cell pellets were treated with 200 \mu g/mL lysozyme and incubated for 30 min to generate spheroplasts. All subsequent steps were carried out at 0–4 °C. Spheroplasts were collected by centrifugation at 10,000 g for 15 min and were re-suspended in 0.03 mol/L Tris buffer (pH 8.1)
containing 3 mmol/L EDTA. The supernatant consisted of the periplasmic fluid, including a peptidoglycan layer. The spheroplasts were disrupted with four 15 s bursts from an ultrasonic processor and centrifuged (2000 g, 10 min) to remove debris and unbroken cells. The resulting supernatant, which consisted of membrane and cytoplasm fractions, was centrifuged at 15,000 g for 150 min. The remaining pellet, which consisted of the membrane envelope, was then dissolved in 15 mL of 50% nitric acid for 30 min.

**X-ray diffraction**

X-ray diffraction patterns of dry, powdered samples of the metal-free control and Co2+-loaded (80 mg/L, 40 h) cells were taken with an Empyrean PANalytical Diffractometer operating at 40 kV using CuKα radiation in the 2θ range from 5° to 80°. Diffraction data from the control and the bacterial biomass were compared to data obtained from other standard cobalt compounds (Joint Committee on Powder Diffraction Standards).

**Results and discussion**

**Effect of metabolic pathways on cobalt bioaccumulation**

Under anaerobic conditions, the removal efficiencies of Co2+ by *R. palustris* were 12.45% and 5.81% in the light and in the dark, respectively. Under aerobic conditions, the removal efficiencies of Co2+ were 50.83% and 89.47% in the light and in the dark, respectively (Figure 1). Removal efficiencies under aerobic conditions were higher than those under anaerobic growth; optimal bioaccumulation occurred under aerobic growth in the dark. *R. palustris* is the most metabolically versatile bacteria known and is capable of changing its metabolism in response to changes in light and oxygen availability [18]. *R. palustris* has also been studied for its ability to biodegrade aromatic rings in the absence of molecular oxygen using an anoxic-sensitive enzyme [25]. It has been suggested that the metabolic pathways that are active within the cell may significantly influence the mechanism of cobalt bioaccumulation.

**Effect of pH on cobalt bioaccumulation**

pH is considered an important environmental factor for bioaccumulation, as it affects cell growth, metal solution chemistry and the ionization state of the cell wall. Figure 2 shows that the maximum biomass and Co2+ removal efficiency (0.78 g and 90.8%, respectively) were obtained at a pH of approximately 6.5. Figure 2 also reveals the positive correlation between removal efficiency and biomass: removal efficiency increased with increasing biomass. High concentrations of H+ likely competed with cobalt ions at cell surfaces, inhibiting the approach of positively charged cobalt ions and decreasing removal efficiencies [26].

**Effect of temperature on cobalt bioaccumulation**

Figure 3 shows that, at 20 °C, *R. palustris* grew extremely slowly, exhibiting a removal efficiency of 11.27%. Similarly, *R. palustris* growth was also significantly inhibited at 40 °C: removal efficiency was 45.58% at this temperature. Maximum biomass (0.78 g dry cell/L) and Co2+ removal efficiency (89.25%) were observed at 30 °C. This result suggests that cobalt removal efficiencies are mainly due to the amount of organism present in the culture. Bioaccumulation processes that depend on

![Figure 1. Effect of metabolic pathways on Co2+ removal efficiency and growth incubated in the enrichment medium with the addition of 80 mg/L Co2+, 30 °C and pH 6.5. The error bars represent the standard deviation at n = 3.](image)

![Figure 2. Effect of pH on Co2+ removal efficiency and growth incubated in the enrichment medium with the addition of 80 mg/L Co2+, 30 °C and aerobic condition in darkness. The error bars represent the standard deviation at n = 3.](image)
cellular metabolism, such as active uptake, would be inhibited by low temperatures. In contrast, high temperatures could affect the integrity of cell membranes and hinder the compartmentalization of cobalt ions [27].

**Effect of co-cations on cobalt bioaccumulation**

The removal efficiency of Co²⁺ by *R. palustris* was 86.8% when cobalt was the only metal ion present. However, Co²⁺ removal efficiencies varied if other cations were also present (Figure 4). In the presence of Fe²⁺, Co²⁺ removal efficiency was slightly increased. Removal efficiencies were not significantly affected by the presence of Cu²⁺, Zn²⁺, Cd²⁺ and Mn²⁺. Interestingly, Co²⁺ removal efficiency decreased to 59.67% from 86.8% in the presence of Ni²⁺. *Rhodococcus rhodochrous* (*R. rhodochrous*) has been reported to behave similarly, showing a preference of Co²⁺ that is unaffected by Mn²⁺ and Zn²⁺ [28]. Rashmi [29] reported that a mutant fungus grown in iron-bearing medium before exposure to a simulated decontamination solution containing 0.033 μmol/L Co²⁺-EDTA exhibited increased or only marginally reduced (<10%) cobalt bioaccumulation capacity despite the presence of a high concentration of iron in the growth medium (initial[Fe/Co] = 2.5 × 10³). The present data suggest that *R. palustris* may have evolved a specific mechanism to tolerate and accumulate high levels of cobalt in hostile environments.

**Effect of initial cobalt concentrations on cobalt bioaccumulation**

As the initial cobalt concentration was increased, the lag time of *R. palustris* also increased while the total biomass decreased under aerobic conditions in the dark (Figure 5). In particular, cultures with an initial metal concentration of 160 mg/L exhibited a lag phase of 25 h and a clear decrease in biomass accumulation (0.5 g/L). This result implies that cell growth was strongly inhibited by the presence of 160 mg/L Co²⁺ in the culture medium. In contrast, in a culture containing 20 mg/L Co²⁺, both the growth curve and lag phase were similar to the control group, indicating no inhibition. The majority of cobalt ions were removed regardless of the initial Co²⁺ concentration (Figure 6). More specifically, cultures with initial Co²⁺ concentrations of 20, 40, 80, 120 and 160 mg/L contained 2.5, 4.62, 9.53, 16.12 and 24.1 mg/L Co²⁺ following culture, respectively. These final Co²⁺ concentrations represented removal efficiencies of approximately 88.9%, 88.45%, 88.08%, 86.56% and 84.9%, and bioaccumulation capacities of 15.24, 37.95, 87.41, 185.61 and 287.18 mg/g, respectively. These data demonstrate that

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**Figure 3.** Effect of temperature on Co²⁺ removal efficiency and growth incubated in the enrichment medium with the addition of 80 mg/L Co²⁺, pH 6.5 and aerobic condition in darkness. The error bars represent the standard deviation at *n* = 3.

**Figure 4.** Effect of co-cations on Co²⁺ removal efficiency incubated in the enrichment medium with the addition of 80 mg/L Co²⁺, pH 6.5, 30 °C and aerobic condition in darkness. The error bars represent the standard deviation at *n* = 3.

**Figure 5.** Effect of initial cobalt concentration on growth incubated in the enrichment medium, pH 6.5, 30 °C and aerobic condition in darkness. The error bars represent the standard deviation at *n* = 3.
although Co\(^{2+}\) bioaccumulation capacity increases with increasing initial Co\(^{2+}\) concentration, Co\(^{2+}\) removal efficiency decreases with increasing Co\(^{2+}\) concentration.

Kinetics of cobalt removal by R. Palustris

To describe the dynamic removal of cobalt by R. palustris, correlation analysis parameters were conducted using a pseudo-first-order kinetic model (Equation (3)) and a pseudo-second-order kinetic model (Equation (4)). These models have been widely applied to many organic material degradation systems and heavy metal removal processes [30–32].

\[
\begin{align*}
\frac{-dC}{dt} &= kC \\
\frac{-dC}{dt} &= k_2 C^2 + k_1 C + k_0 
\end{align*}
\]

By separation of variables and integration with Equation (5), the relationship between Co\(^{2+}\) concentration and removal time was obtained:

\[
C = \frac{h(C_0 - q) - q(C_0 - h)e^{kt}}{(C_0 - q) - (C_0 - h)e^{kt}} \\
p = \sqrt{k_1^2 - 4k_0k_2} \\
q = \frac{(-k_1 + p)}{2k_2} \\
h = \frac{(-k_1 - p)}{2k_2}
\]

Table 1. Pseudo-first-order model kinetic equations and kinetic parameters of the removal for different concentration of cobalt by growing R. palustris.

| Co\(^{2+}\) (mg/L) | \(k\)   | \(R^2\) | \(C = C_0e^{-kt}\) |
|-------------------|---------|---------|-------------------|
| 20                | 0.0540  | 0.9184  | 24.53e\(^{-0.0540t}\) |
| 40                | 0.0546  | 0.8881  | 49.96e\(^{-0.0546t}\) |
| 80                | 0.0500  | 0.8905  | 98.00e\(^{-0.0500t}\) |
| 120               | 0.0429  | 0.8639  | 146.92e\(^{-0.0429t}\) |
| 160               | 0.0264  | 0.7848  | 181.06e\(^{-0.0264t}\) |

where \(C_0\), \(C\), \(k\), \(k_0\), and \(k_2\) are the initial concentration of cobalt, the concentration of cobalt at time \(t\) (mg/L), contact time (h), the rate constant of pseudo-first-order, constant and coefficient of \(C\) and \(C^2\) in a pseudo-second-order equation, respectively.

Table 1 indicates that the pseudo-first-order kinetic model poorly fits the experimental data, with low correlation coefficients (\(R^2 = 0.7848–0.9184\)). It is obvious that the pseudo-second-order kinetic model better fits the removal process. From Table 2, the values of \(q\) (22.5324, 44.2804, 87.2258, 117.1875 and 153.83), which represented remaining cobalt concentrations, agreed closely with the experimental values (20, 40, 80, 120 and 160 mg/L), also closely agreed with the experimental values. The values of \(p\), which represented the maximum removal speed of cobalt, decreased from 0.2753 to 0.1871 h\(^{-1}\) as the initial concentration of cobalt increased. Values for \(k_2\) were negative (−0.0108, −0.0069, −0.0031, −0.0024 and −0.0014). Such negative values could be interpreted as the inhibitory effect of cobalt on R. palustris; the low absolute values of \(k_2\) suggest minimal inhibition. Values for \(k_1\), which are related to removal efficiencies, were positive (0.2672, 0.3358, 0.2979, 0.3201 and 0.2437). Finally, values for \(k_0\) were negative, which can be explained by competitions within the bacterial population for the limited amount of substrate in the medium.

Cell compartmentalization

The amount of cobalt sequestration in R. palustris led us to investigate the compartmentalization of cobalt in various cell sectors. It was shown that, following Co\(^{2+}\)
exposure (80 mg/L, 40 h), cobalt was distributed between the four cell compartments in *R. palustris*. Cobalt in the cell surface, periplasmic space, membrane fraction and cytoplasm represented 5.72%, 57.37%, 27.95% and 8.82% of the total cobalt taken up, respectively (Figure 7). The result differed from a previous report, in which the removal of heavy metals was primarily attributed to the cell surface [23]. Guo et al. [33] reported similar results, noting that the majority of the removal and transformation of Cd (II) and Pb (II) ions by EB L14 occurred in the membrane fraction. Sar [34] also reported that 88% of Ni2+ was restricted to the periplasm and cell membrane by *Pseudomonas aeruginosa* in combination with phosphoryl groups.

The distribution and uptake of Co2+ in *R. palustris* indicates that cobalt deposition primarily occurred at the membrane and in the periplasm, implying that intracellular accumulation plays a significant role in cobalt sequestration. Unknown components involved in Co2+ binding in the periplasmic space and membrane fraction of *R. palustris* remain. It seems that surface adsorption by electrostatic attraction and functional group binding with Co2+ only play minor role in cobalt uptake. Such peripheral sequestration of toxic cations exterior to the cytoplasm has been suggested to protect growing cells by limiting the amount of Co2+ transport into the cytoplasm.

**X-ray diffraction analysis**

The chemical nature of cellular cobalt was ascertained via X-ray diffraction analysis of powdered bacterial biomass. The control biomass, which lacked any distinct peaks, is characterized by an amorphous nature (Figure 8(a)). However, Co2+-loaded biomass exhibited several distinct peaks, indicating that cobalt crystals were deposited in the cells (Figure 8(b)). In comparison with X-ray diffraction (XRD) standards available in databases, the diffraction pattern of Co2+-loaded biomass corresponded to that of cobalt phosphate octahydrate (Joint Committee on Powder Diffraction Standard (JCPDS) card 01–0121). Such observations strongly suggest that Co2+ interacted with phosphate groups in *R. palustris* to form cobalt phosphate octahydrate crystals. In a similar fashion, *P. aeruginosa* accumulated uranium due to precipitation of intracellular crystalline U-phosphate compounds [35]. The formation of nickel phosphides and carbides was also reported, primarily in the cell envelope of *P. aeruginosa*, indicating the predominant role of the phosphoryl and carboxyl/carbonyl groups in cell wall/membrane components for metal sequestration [34].

**Conclusion**

The optimal pH, temperature and metabolic pathway for cobalt bioaccumulation were 6.5, 30°C and aerobic growth in the dark, respectively. A pseudo-second-order kinetic model was able to describe such a cobalt removal system under optimal culture conditions. Cu2+, Zn2+,...
Cd$^{2+}$, Mn$^{2+}$ and Fe$^{2+}$ did not significantly influence cobalt removal efficiencies, while Ni$^{2+}$ did. Furthermore, cell compartmentalization analysis revealed that 85.32% of the accumulated cobalt was distributed between the periplasm space and the membrane, implying that intracellular accumulation plays a significant role in cobalt sequestration. The accumulated cobalt was determined to be cobalt phosphate octahydrate by X-ray diffractometry. This study demonstrates that *R. palustris* has a great potential for the removal of cobalt from industrial wastewaters.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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