Biosorption of heavy metal ions by *Citrobacter freundii*

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Summary

The problem of environment pollution of excess heavy metal ions in water bodies is rampant and becoming more serious. However, current methods to treat this problem have been deemed to be inefficient and time consuming. Hence for this project, the use of the bacterium, *Citrobacer freundii*, in the biosorption of heavy metal ions was explored, as the adsorption process occurs at a much faster rate. Through experimentation it was found that *C. freundii* was using a non-living mechanism to adsorb heavy metal ions and hence the mechanism responsible for this adsorption was extracted and explored for its applications.

Abstract

Heavy metal pollution in the environment is mainly caused by illegal disposal of industrial wastes. These ions include lead, cadmium, zinc, mercury, manganese, copper (II) and iron (III). This project explores the effectiveness of using the Gram-negative bacterium, *Citrobacter freundii*, in biosorption of these ions. It was found that *C. freundii* was most efficient at adsorbing copper (II) and iron (III) ions. Alginate immobilized *C. freundii* was able to adsorb copper (II) ions form contaminated water samples, while *C. freundii* exposed to UV light for 40 s showed greatest efficiency of adsorption. Furthermore, *C. freundii* killed by freeze thaw cycles could also adsorb copper (II) ions. Thus adsorption could take place without the introduction of cells into a polluted environment, thus maintaining a balance in the ecosystem. *C. freundii* lysate containing soluble cell components showed significant adsorption of copper (II) ions. This suggested the involvement of an intracellular protein which chelated the copper (II) ions.

Rationale

The problem of environment pollution of excess heavy metal ions in water bodies is rampant. Alloway (1995) reported that a method of treatment of such polluted environments includes utilizing metalotolerant plants to adsorb the heavy metal ions. However, this method of treating the polluted terrestrial or aquatic environment requires rather long treatment durations and thus deemed as inefficient. Moreover, the plants show low growth rates. Hence, in this project, the use of the bacterium, *C. freundii* in the biosorption of heavy metal ions is explored.

The objectives of this project are to investigate the effect of immobilization of *C. freundii* cells on biosorption of copper (II) ions from contaminated water samples and the effects of short durations of ultraviolet (UV) radiation on growth and efficiency of adsorption of copper (II) ions. This project also aims to investigate the ability of adsorption of copper (II) ions by *C. freundii* cells treated by freeze-thaw cycles and the mechanism by which *C. freundii* adsorbs these ions.

We hypothesise that:

- Immobilized *C. freundii* cells are able to adsorb ions.
- *C. freundii* cells are able to adsorb ions more efficiently after exposure to UV light.
- *C. freundii* cells killed by the freeze-thaw cycles are still able to adsorb copper (II) ions.
- After cell lysis, the soluble intracellular components and the cell membrane and cell wall will exhibit different degrees of adsorption of copper (II) ions.

Background

With current rapid industrialization in developing countries, there have been numerous reports of heavy metal pollution in the environment by illegal disposal of industrial waste. Duruibe *et al*.

(2007) suggested heavy metal ions from pollutions include lead, cadmium, zinc, mercury, manganese, arsenic, silver, chromium, copper and iron (III). Leo (2000) indicated that natural levels of heavy metals, especially those from aquatic ecosystems, have significantly increased in the last decades simultaneously with the high development of industrial activities and urban development. According to Alluri *et al.* (2007), such heavy metal pollutions that are non-biodegradable has devastated many biological environments, and posed many health risks to the human population.

Alluri *et al*. (2007) and Duruibe *et al.* (2007) reported that the harmful effects of heavy metal ions on humans range from damage to the kidneys, the nervous, reproductive and cardiovascular systems and in excessive amounts, even cause death. Metal ions can also bring about harmful effects to plants. According to Gallego *et al.* (1996), metal ions are capable of decreasing certain enzymes present in plants, weakening the plant as a result of oxidative damage.

Current methods of removing heavy metal ions include their hyperaccumulation in plants. However, this method has been proven to be impractical due to the small size of these metal hyperaccumulating plants (Prasad and Freitas, 2003). The harvesting of these plants take a lot of time as they tend to be slow growing plants and produce little biomass (Lasat, 2000). In response to this, Alluri *et al.* (2007) has reported that biosorption of heavy metal ions by some bacterial species may prove to be a better treatment alternative towards heavy metal pollutions.

From past research conducted in 2009, it was found that *C. freundii* was able to adsorb copper (II), iron (III), zinc and manganese ions, resulting in a decrease of 26.8%, 28.9%, 13.2% and 11.1%, respectively, of metal ion concentrations. Copper (II) ions least inhibited the growth of *C. freundii*. *C. freundii* also adsorbed copper (II) ions at the highest rate in aqueous solution of pH of 5.56 and 7.65, among the five pH values tested (4.65, 5.65, 6.65, 7.65, 8.65).

Methods

Immobilisation of *C. freundii* in alginate beads

C. freundii cell suspension was added to equal volume of sodium alginate solution. This was added dropwise to calcium chloride solution to form alginate beads. The control consisted of adding sodium alginate solution without cells dropwise into calcium chloride solution. The following set-ups were prepared: a control consisting of the contaminated water sample without beads (a comparison to find out if concentration of metal ions decreases in the absence of cells); a second control of 30 alginate beads without cells in a contaminated water sample containing copper (II) ions, and a test set-up which included 30 *C. freundii* alginate beads in the water sample. All set-ups were incubated in an orbital shaker at 30°C for 48 h. The final ion concentration was tested using the colorimeter.

Investigation of effect of UV on efficiency of adsorption of copper (II) ions by *C. freundii*

Based on the principle of hormesis, the application of stressor agents in low doses will stimulate a subjective beneficial response, leading to higher growth rate (Baldwin and Calabrese, 2003). The relationship between the application of the dosage of stressor agent and the growth rate response is depicted in the typical hormetic curve which has an inverted U-shape. Therefore it is possible that *C. freundii* when exposed to UV radiation will grow at a higher rate and thereby result in an increased rate of biosorption of copper (II) ions. As such, *C. freundii* cells were exposed to UV light of 302 nm for short durations.

C. freundii cells was placed on a Petri dish and exposed to UV light from a UV transilluminator for 20 s, 40 s and 60 s. The following set-ups were prepared: a control of LB broth with 50 ppm copper (II) ions without cells; a second control with non-UV-exposed *C. freundii* cells with 50 ppm copper (II) ions in LB broth; and 3 test set-ups consisting of UV-exposed cells for 20, 40 and 60 s, respectively, with 50 ppm copper (II) ions in LB broth. The set-ups were incubated at 30°C for 48 h in the orbital shaker. The final copper (II) ion concentration was then determined.

Test for adsorption of ions by dead *C. freundii* cells

C. freundii was first inoculated in 30 ml LB broth and incubated at 30°C for 24 h. 1 ml of *C. freundii* was each transferred to 9 ml LB broth and incubated for 24 h. The cultures were centrifuged at 8000 rpm for 10 min and resuspended in 1 ml LB broth to undergo the freeze-thaw cycle. This comprised of three cycles of exposing cells to -86°C for 10 min in an ultralow freezer, followed immediately by exposure to 65°C for 10 min in a water bath. There were three set-ups, each prepared in triplicates: a control with 50 ppm copper (II) ions without *C. freundi*i; a test set-up with 50 ppm copper (II) ions with live *C. freundii*; and another test set-up with 50 ppm copper (II) ions with freeze-thawed *C. freundii* cells.

Investigation of mechanism by which *C. freundii* adsorbs heavy metal ions

B-PER reagent was added to *C. freundii* cells at a volume of 4 times the mass of the cell pellet. This was incubated at room temperature for 10 min for cell lysis to occur. The contents were centrifuged at 13000 rpm for 4 min. The supernatant which was the cell lysate was collected.

The following set-ups were prepared: a control with 50 ppm copper (II) ions and B-PER without *C. freundii* (a comparison to find out if concentration of metal ions decreased in the absence of cell pellet and lysate; a test set-up with 50 ppm copper (II) ions with cell lysate containing soluble intracellular proteins (supernatant); another test set-up with 50 ppm copper (II) ions with insoluble cell components (pellet) such as the cell wall and cell membrane. Triplicates were carried out for all set-ups. They were incubated in the orbital shaker for 48 h at 30°C.

Immobilisation of *C. freundii* lysate in alginate beads

C. freundii cells were exposed to -86°C for 10 min, and thawed at 65°C for 10 min. The cells were then sonicated for 20 min. The cell suspension was then centrifuged, and the supernatant (containing the cell lysate) and the pellet (containing insoluble cell wall and cell membrane) were collected. The following set-ups were prepared: a control of 50 ppm copper (II) ions; a second control of alginate beads containing LB broth; and a test set-up with *C. freundii* lysate immobilized in alginate beads in 50 ppm copper (II) ions. They were incubated at 30°C for 48 h. The final concentration of copper (II) ions was determined.

Results

Effect of immobilization of cells on adsorption of ions

There was a decrease in concentration of copper (II) ions in both test set-ups which contained blank alginate beads and alginate beads containing *C. freundii* as compared to the control.

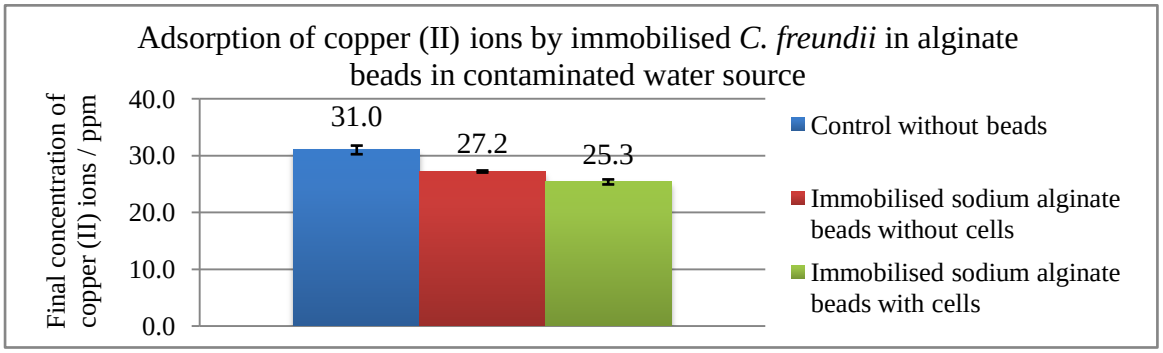

Figure 1: Graph showing adsorption of copper (II) ions in contaminated water source by immobilized *C. freundii* cells

There was a significant decrease in the concentration of copper (II) ions between the test and control set-up ($p=0.006$ for alginate beads with *C. freundii*). Hence *C. freundii* was proven to be able to adsorb copper (II) ions while in alginate beads, resulting in a mean decrease of 18.4% of copper (II) ions. The blank alginate beads also resulted in a mean decrease in 12.3% of copper (II) ions, probably due to the chelation of ions by alginate. The results are summarized in Figure 1.

Investigation of effect of UV on efficiency of adsorption of copper (II) ions by *C. freundii*

There was a significant decrease in the concentration of copper (II) ions for both non-UV-exposed and UV-exposed cells. *C. freundii* exposed to UV radiation for 40 s adsorbed the most heavy metal ions, showing a mean decrease of 31.4% in copper (II) ions, compared to 18.4% observed with the non-UV-exposed *C. freundii*. Hence *C. freundii* was proven to be able to adsorb copper (II) ions more efficiently after UV radiation.

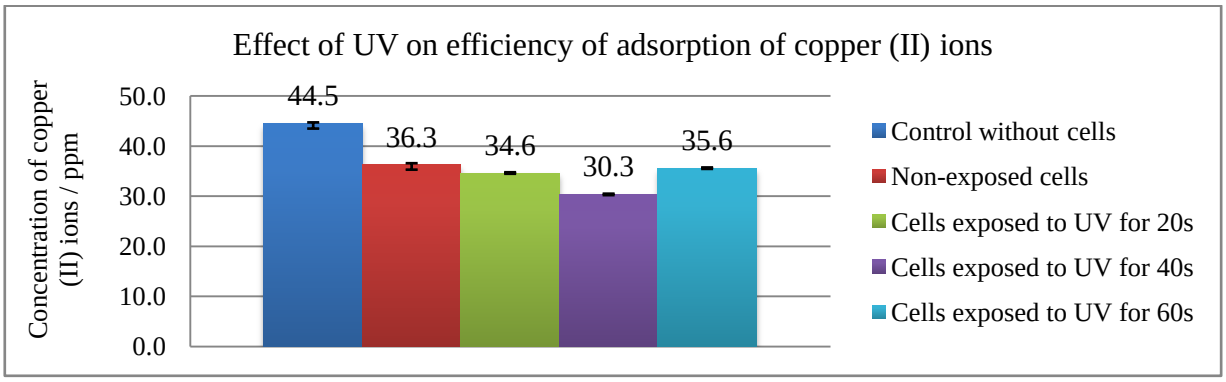

Figure 2: Graph showing the effect of UV on the efficiency of adsorption of ions by *C. freundii*

The number of colonies increased significantly when *C. freundii* was subjected to 40 s of UV radiation. This duration of UV exposure probably induced the strongest hormetic response resulting in the highest growth rate. However, when *C. freundii* was subjected to UV for 60 s, the number of *C. freundii* colonies decreased significantly, suggesting that 60 s excessive in dosage and was lethal to some *C. freundii* cells.

Adsorption of heavy metal ions by dead cells

There was a significant decrease in the concentration of copper (II) ions in both test set-ups containing live and dead cells, by 31.9% and 33.0%, respectively (Figure 3). This observation proved that non-living *C. freundii* cells were also able to adsorb copper (II) ions.

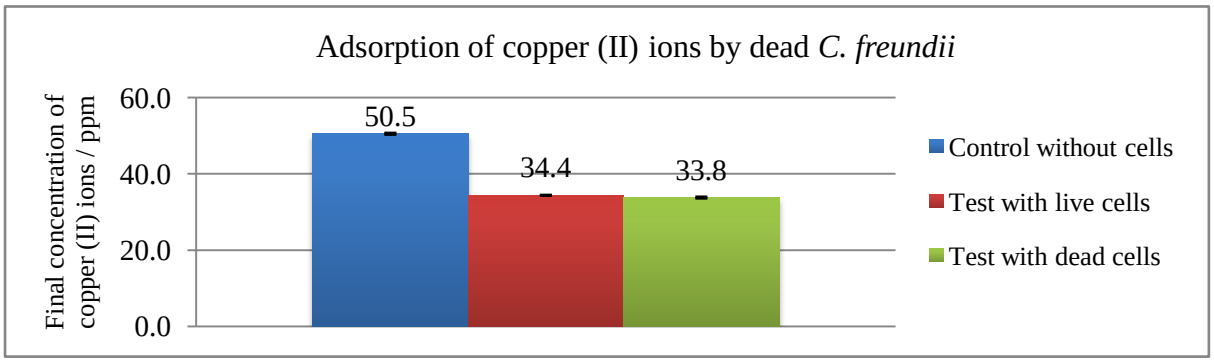

Figure 3: Graph showing the adsorption of copper (II) ions by freeze-thawed *C. freundii* cells

Investigation of mechanism by which *C. freundii* adsorbs heavy metal ions

Gram staining revealed that B-PER has successfully caused the lysis of cells as the pellet consisted of cell debris without any regular shape. There was almost no decrease in the concentration of copper (II) ions in the set-up with the pellet (containing debris of cell walls and cell membranes) and a significant decrease in the set-up with the supernatant (containing the intracellular soluble cell components) as compared to the control. This is shown in Figure 4.

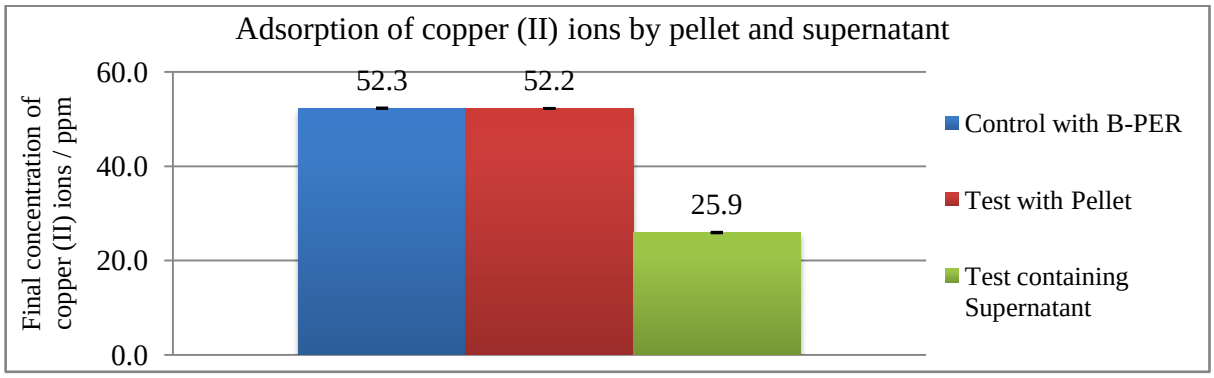

Figure 4: Graph showing the adsorption of copper (II) ions by the cell lysate containing soluble components of the cells

The test set-up containing the supernatant resulted in a significant decrease in 50.4% of copper (II) ions as compared to the control, with a t-test p-value of 1.817×10^{-8}. However the p-value of the set-up containing the pellet, compared to the control, was 0.3868, showing that there was no significant adsorption. This suggests that the soluble cell components, instead of the cell walls or cell membranes that possibly bound to the ions.

Immobilisation of *C. freundii* lysate in alginate beads

Alginate beads without cell lysate showed the ability to adsorb 15.3% of copper (II) ions, while the beads which contained immobilized *C. freundii* lysate had adsorbed 67.7% of copper (II) ions. This result supported the previous set in that the soluble components of the cell lysate had the ability to bind to the ions. There was a significant decrease in the ion concentration in the presence of the cell lysate below that of control without cells (p=0.0006) and that of beads with LB (p=0.003), hence proving that *C. freundii* lysate on its own had the ability to adsorb a significant amount of copper (II) ions. The results are shown in Figure 5.

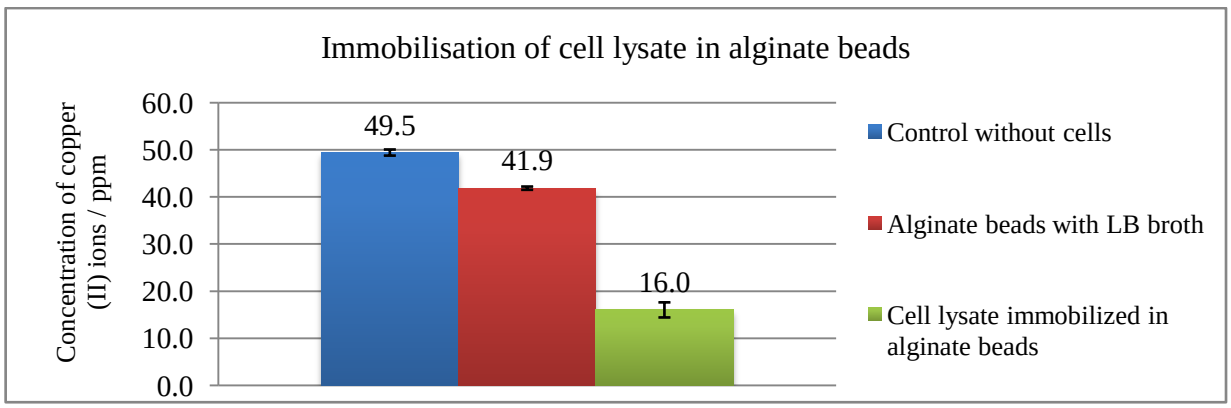

Figure 5: Graph showing the adsorption of copper (II) ions by cell lysate containing soluble cellular components

Conclusions and Discussion

The introduction of *C. freundii* alginate beads into a contaminated water source resulted in a decrease of 18.4% of copper (II) ions. However, there was also a decrease observed when blank alginate beads without cells were used as a control. According to Eliaz *et al.* (2007), alginate binds to heavy metal ions, leading to a decrease in the number of free copper (II) ions in an aqueous solution. Thus, it can be concluded that when *C. freundii* was immobilized in alginate beads, a percentage of decrease in the concentration of copper (II) ions resulted due to copper (II) ions binding to the alginate beads. However, since beads containing *C. freundii* resulted in a greater and significant decrease in the concentration of copper (II) ions, it can be concluded that *C. freundii* has the ability to adsorb copper (II) ions while entrapped in sodium alginate beads.

The efficiency of adsorption was increased the most by an exposure to UV radiation for 40 s, thus resulting in a mean decrease of 31.9% copper (II) ions. This was probably due to the highest growth rate of *C. freundii* after exposure to UV rays for 40 s, based on a hermetic response to a stressor agent such as UV.

C. freundii cells killed by the freeze-thaw cycle were still able to adsorb copper (II) ions. There was no significant difference between the amount of ions adsorbed by the dead cells and live cells, suggesting that the mechanism adopted by *C. freundii* cells does not require active transport. As documented in Alluri *et al.* (2007), the possible mechanisms for biosorption include cell surface adsorption where bonds are formed due to electrostatic attraction between carboxyl groups on the cell wall of bacteria and the metal ions; or by intracellular accumulation of metal ions which are adsorbed to proteins after ions are transported across the cell membrane. In our experiments, there was no significant decrease in the concentration of copper (II) ions with the pellet containing debris of cell wall and cell membrane after B-PER treatment. Thus the mechanism of biosorption is not likely to be by cell surface biosorption where copper (II) ions form bonds with carboxyl groups of cell walls. Furthermore, the supernatant containing soluble proteins resulted in 50.4% decrease in copper (II) ions. This suggests that the mechanism of biosorption is likely to be intracellular accumulation of copper (II) ions by binding to a protein in the cell. Lastly, immobilized *C. freundii* lysate was found to have adsorbed 67.6% of copper (II) ions, further proving that *C. freundii* adsorbs copper (II) ions by transporting copper (II) ions across the cell membrane, followed by binding to an intracellular protein.

The results of this study can be applied in bioremediation of environments polluted with heavy metal ions. The introduction of non-living cells or cell lysates of *C. freundii* into these environments ensure that the balance of the ecosystem is not disrupted. Moreover, *C. freundii* have short generation time and high growth rates, so it is more likely to show greater efficiency in

biosorption than plants. Further work involves the investigation of the adsorption of other heavy metal ions such as lead. Other forms of immobilization of *C. freundii* cells can also be explored.

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