Bioremediation of soluble heavy metals with recombinant *Caulobacter crescentus*

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Abbreviations: 6His, hexahistidine; CDW, cell dry weight; EDTA, ethylene diaminetetraacetic acid; S-layer, surface layer

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To achieve one-step separation of heavy metal ions from contaminated water, we have developed a novel bioremediation technology based on self-immobilization of the *Caulobacter crescentus* recombinant strain JS4022/p723-6H, which overexpresses hexahistidine peptide on the surface of the bacterial cells and serves as a whole-cell adsorbent for dissolved heavy metals. Biofilms formed by JS4022/p723-6H are effective at retaining cadmium from bacterial growth media or environmental water samples. Here we provide additional experiment data discussing the application potential of this new technology. Supplementation of calcium to the growth media produced robust JS4022/p723-6H cells by alleviating their sensitivity to chelators. After growth in the presence of 0.3% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, double the amount of JS4022/p723-6H cells survived the treatment with 2 mM EDTA. Free cells of JS4022/p723-6H effectively sequestered 51% of the total cadmium from a Lake Erie water sample at pH 5.4, compared to 37% retrieved by the control strain. Similar levels of adsorption were observed at pH 4.2 as well. Cells of JS4022/p723-6H were tolerant of acid treatment for 90 min at pH ≥ 1.1 or 120 min at pH ≥ 2.5 , which provides an avenue for the convenient regeneration of the bacterial cells metal-binding capacity with acidic solutions. Designs of possible bioreactors and an operation system are also presented.

Introduction

The term heavy metal is often used to refer to metallic chemical elements of high density and which show toxicity to living organisms. Toxicity is not a defining property of heavy metals; indeed some heavy metals are nutritive at very low levels but are toxic at higher concentrations. For example copper is an essential micronutrient for life due to its convenient redox potential when bound to protein, but excessive copper generates highly reactive hydroxyl radicals, which could cause lipid peroxidation, enzyme inactivation and DNA scission.¹ Other heavy metals such as mercury and lead have no known beneficial effects to organisms. For a long time, cadmium was thought only to be toxic, but interestingly, the way it incurs metabolic damage can also support life under certain conditions. Cadmium is chemically similar to zinc and can inactivate many enzymes by replacing the essential zinc ion at their active sites. A recent study demonstrates, however, a marine diatom expresses a Cd-specific carbonic anhydrase used for concentrating inorganic carbon under Zn limiting conditions as may be experienced in some oceanic regimes.² Despite the positive impacts some heavy metals might have to living organisms, accumulation of elevated levels of heavy metals is deleterious. For humans, exposure to high levels of toxic metals over time may result in a variety of symptoms, including brain damage, liver failure, cancer and even death. Recent studies have also linked heavy metals to autoimmune disease³ and birth defects.^{4,5} Although

heavy metals are natural components of the Earth's crust, human industrialization has released excessive amounts into the environment. Water from many freshwater locales is no longer safe to drink, mercury is bioaccumulated in fish tissues, and lead and cadmium are being detected in the hair of wild animals.⁶ The toxic and ecotoxic effects of these metals have alarmed more and more nations to adopt stringent regulations governing the release of heavy metals to the environment. In spite of this, the toxic metals that have ended up in the environment cannot be degraded or destroyed, and they may bioaccumulate in the food chain. The demands for safe and effective ways to retrieve the metal contaminants from the environment are urgent.

Bioremediation approaches have received growing attention in recent years because of their potential for providing a cost-effective way for heavy metal remediation, especially at dilute concentrations, where traditional technologies such as precipitation, ion exchange and reverse osmosis are either too expensive or not sufficient to achieve desirable results. Qualities of a good heavy metal bioremediation agent would be: (1) safe to humans and the environment, (2) easy to cultivate, (3) tolerant to accumulated heavy metals, (4) readily separated from the treated water, and (5) conveniently regenerated after metal sequestration. The bacterium *Caulobacter crescentus* offers satisfactory solutions to each of the above requirements. *C. crescentus* is nonpathogenic to humans and other organisms, and they thrive in nutrient-limiting environments. Genes involved in the degradation of aromatic compounds and heavy metal resistance have been identified.^{7,8} The surface layer (S-layer) protein of the bacterium allows heavy-metal-binding peptides to be anchored on the outermost surface of the cell for immediate access to environmental heavy metal ions. Overexpression of metal-binding peptides, also known as affinity peptides, on the surface of microbial cells increases the rate and capacity of the adsorption while minimizing the toxicity to host cells. This also makes the regeneration process more convenient because the majority of the metals are sorbed to the cell surface. In addition, mature *Caulobacter* cells can immobilize themselves to solid surfaces

through an adhesive holdfast found at the tip of their stalk-like appendage, lending them as ideal candidates for construction of biofilm-based remedial reactors.

We have reported the construction of a recombinant *C. crescentus* strain JS4022/p723-6H with hexahistidine peptides (6His) inserted at a permissive site of the S-layer protein RsaA.⁹ Because the engineered S-layer fusion protein RsaA-6His is displayed at the cell surface, strain JS4022/p723-6H can be directly used as a whole cell adsorbent for sequestration of dissolved heavy metals from water. When an appropriate amount of bacterial biomass is used, JS4022/p723-6H can remove 97–99.9% of the total dissolved cadmium, as opposed to less than 20% achieved by the control strain JS4022/p4ArsaA(723Δ)GSCCA, which expresses RsaA with a cloning linker inserted at the same permissive site. The specific recognition between 6His and Cd(II) requires as little as 15 min. More recently we have confirmed that self-immobilized JS4022/p723-6H cells evenly coat the surfaces of a glass microscopy coverslip with two-dimensional monolayer biofilms. The biofilms are equally effective at retrieval of cadmium ions from bacterial growth medium as well as water samples collected from Lake Erie.¹⁰ The utilization of the two practical techniques, cell surface display and self-immobilization, has allowed complete separation of heavy metal ions from contaminated water in a single step without the need of a centrifuge or column. This essay supplies additional data and comments regarding a few aspects that are important to the deployment of this promising technology.

Protection of Calcium Against Ethylene Diaminetetraacetic Acid (EDTA)

Our previous report discovered that EDTA causes damage to JS4022/p723-6H cells, a common phenomenon observed with gram negative bacteria whose outer membranes are thought to be stabilized by divalent ions, particularly calcium.¹¹ Cells exposed to EDTA lose their calcium to the chelator and suffer various degrees of lysis, which could potentially undermine the remedial value of this gram negative strain as many

types of wastewater might contain chelators like EDTA. Here we present evidence showing that supplementation of calcium to the growth medium is able to buffer the adverse effects of the chelator. The recombinant strains were cultivated overnight at 30°C in PYE selection medium (0.2% peptone, 0.1% yeast extract, 0.01% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 $\mu\text{g mL}^{-1}$ chloramphenicol) supplemented with or without 0.3% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. Cells were harvested by centrifugation at 5,000 *g* for 10 min, washed once in 20 mM Tris·Cl buffer (pH 7.5), and resuspended in the same buffer to reach a final $\text{OD}_{600\text{nm}}$ around 1.0. After the addition of 2 mM EDTA, the cells were gently rocked at room temperature and the change in optical density was monitored for up to 100 min. As shown in Figure 1, during 100 min of exposure to EDTA, the survival rate of the cells grown in plain PYE dropped continuously. By the end of the test, only 32% and 54% of the original cells survived for JS4022/p723-6H and the control strain, respectively. Extending the exposure time is expected to augment the degree of cell lysis. In contrast the population of cells cultivated in the presence of additional calcium remained constant following a slight decrease during the initial 25 min. For JS4022/p723-6H, the number of cells that were able to survive to the end of the treatment doubled from 32 to 65%. The protective effect was also prominent for the control strain, whose survival rate increased from 54 to 90%. Supplementation with calcium might reduce the nonspecific binding of heavy metals because the outer membrane of the bacteria would be less negatively charged. However, the specific recognition between polyhistidine and heavy metals is unlikely to be affected. It has been demonstrated that Ca^{2+} , Mg^{2+} , Fe^{2+} and Al^{3+} do not interfere with the specific interaction between Cd and polyhistidines, either individually or as a mixture.^{9,12}

Removal of Cadmium from Lake Erie Water Samples at Different pH

In order to evaluate the application potential of the strain JS4022/p723-6H, we tested it with an environmental water

sample collected from the central basin of Lake Erie at Environment Canada Station 84 (41° 56.07 N, 81° 39.11 W, depth: 22.3 m). The influence of pH on heavy metal sequestration was investigated in detail. The lake water was sterilized using membrane filtration, acidified with concentrated HCl when needed, and spiked with CdCl₂ to reach a final concentration of 1.0 ppm Cd(II) prior to use. Bacterial cultures grown overnight were harvested by centrifugation and were resuspended in the above treated lake water. After incubation at 30°C with gentle shaking at 100 rpm for 30 min, the cadmium content remaining in the water was measured with an atomic absorption spectrometer. At the natural pH of the water sample (~pH 8), the adsorption of cadmium was dominated by non-specific interactions with only marginal specific recognition between 6His and Cd(II) observed (data not shown). One of the possible reasons could be the cadmium ions are clustered with other compounds, such as dissolved organic carbon in the lake water and are unavailable to the 6His peptides. We reasoned that lowering the pH of the water would free the metal ions and enhance the specific recognition, a hypothesis which was supported by our results. At pH 5.4, each gram of JS4022/p723-6H (cell dry weight, CDW) could sequester 1.9 mg of Cd(II) (51% of the total) from the water, as opposed to 1.4 mg (37% of the total) accumulated by the control strain (Fig. 2). The levels of removal agree precisely to our previous study using biofilms of the strains, where JS4022/p723-6H sequestered 44~51% of the total spiked cadmium and the control strain retained 37~42%.¹⁰ This provides direct evidence that self-immobilization of *Caulobacter* cells to solid surfaces does not interfere with their bioadsorptive function. Because of the unique monolayer feature of the biofilms formed by *Caulobacter* strains, we previously predicted that immobilized JS4022/p723-6H cells would function equally well as free cells.¹⁰

Similar levels of removal were also observed at pH 4.2 (Fig. 2). When the acidity was lowered further to pH 3.0, however, the amount of the metal bound to either strain dropped by ~60%, resulting in 0.74 and 0.50 mg g⁻¹ CDW removal for

Figure 1. Supplementation of 0.3% CaCl₂·2H₂O renders the recombinant strains resistant to EDTA. RsaA-6His represents strain JS4022/p723-6H, and RsaA(723) refers to the control strain JS4022/p4ArsaA(723Δ) GSCCΔ.

Figure 2. The removal of cadmium from Lake Erie water samples by free cells of JS4022/p723-6H and JS4022/p4ArsaA(723Δ) GSCCΔ depicted as percentages (A) and absolute mass amount (B). RsaA-6His represents strain JS4022/p723-6H, and RsaA(723) refers to the control strain JS4022/p4ArsaA(723Δ) GSCCΔ.

Figure 3. Stability of JS4022/p723-6H under acidic conditions.

JS4022/p723-6H and the control strain, respectively (Fig. 2). The competition of hydrogen ions for binding to peptides at low pH makes it possible to regenerate the binding sites of the peptides through acid washes. The toxic metals can then be collected at a much higher concentration whereas in a dramatically reduced volume, rendering the waste easier and safer to handle. Previous data indicate that Cd dissociates completely from most affinity peptides at pH 3–5.5.¹³ Hexahistidine peptide seems to have a higher than usual affinity to Cd since it still specifically recognizes Cd at pH 3.0 (Fig. 2). Applying washing solutions with a lower pH should result in better regeneration outcomes. The question is, would JS4022/p723-6H survive a harsher treatment?

Stability of JS4022/p723-6H Under Acidic Conditions

Bacterial cultures were grown overnight, harvested by centrifugation, and resuspended in 10 mM citrate buffer of varying pH to reach final OD_{600 nm} around 1.0. The citrate buffer was chosen due to its excellent buffering capacity under acidic conditions. The optical density of the culture was monitored for 2 hours at 600 nm. As plotted in Figure 3, cells of JS4022/p723-6H experienced no apparent destruction within 2 hours from pH

2.5 through 5.5, although slight cell lysis was observed after 90 min exposure at pH 1.1. The control strain exhibited identical tolerance levels as did JS4022/p723-6H (data not shown). These results suggest regeneration of the metal-laden bacterial cells may be achieved with acidified buffer at pH <3. It is worthwhile to mention that diluted acid at pH 2 to 2.5 has been used to release lead adsorbed to microbes.¹⁴

Development of Remedial Bioreactors

For the technology to be applied for bioremediation purposes, proper bioreactors are needed to perform the tasks. A bioreactor is a system that supports and contains biological agents such as microorganisms during a designated process. Bioreactors are operated with defined and controllable environmental parameters and are therefore an efficient way of exploiting applications of biological systems. There are many different types of bioreactors available for remediation of heavy metals. Traditionally, immobilized microbial bioreactors have used microorganisms entrapped in an external matrix such as calcium alginate, carrageenan, polyurethane, and other polymeric membranes. Although high biomass concentration can be reached, it is very difficult to regenerate these types of reactors. Considering

Caulobacter's unique capability of forming stable and uniform monolayer biofilms, a spiral-sheet bioreactor or a fiber brush bioreactor should be well-suited for the intended remedial applications. Both types of reactors provide large surface areas for attachment of microbial cells, and the flow routes in the spiral channels or between the brush fibers allow sufficient microbe-metal ion interactions.

In a spiral-sheet bioreactor, microbial biofilms formed on biosupporting sheets are wound in a spiral configuration (Fig. 4A). The spiral sheet may be supported between stainless steel plates and a plastic housing with provision of inlet, outlet and drain. The space between two adjacent layers may be enforced by projecting ribs, creating the channel for flow of liquid. Spiral-sheet bioreactors have been widely used in a variety of microbial biotechnology applications, including production of lactic acid from cheese whey¹⁵ and biosorption of lead from synthetic waste streams.¹⁴ In a fiber brush bioreactor, biosupporting fibers are tied to a central core giving the configuration of a brush, which can greatly increase the cell loading amount and thus the overall bioactivity of the reactors (Fig. 4B). A small brush, 2.5 cm in outer diameter and 2.5 cm long, made of carbon fibers of 7.2 μm in diameter has an estimated surface area of 0.22 m² or 18,200 m²/m³-brush volume.¹⁶ Such fiber brushes have been successfully used as anodes for increased power production in microbial fuel cells.¹⁶ Multiple brushes can be easily bundled and used in larger-size bioreactors. A complete bioremediation system may include a bioreactor, pumps and valves, water sample vessels, and a monitoring unit to control pH and temperature as illustrated in Figure 4C. Bioaccumulation can be conducted by circulating heavy metal waste through the reactor until no more metal adsorption is observed from the outlet.

In situ recovery of a heavy metal could be performed by circulating acidic solutions. As the biofilms of *Caulobacter* can self-heal from stress and mechanical abrasion,¹⁷ damage to the biofilms caused by acid washes may be repaired by supplying bacterial growth media to the reactor. Because of its biological origin, any water processing devices utilizing bacterial

Figure 4. Schematic diagrams of (A) a spiral-sheet bioreactor immobilized with *Caulobacter* cells, adapted from Shahbazi et al.¹⁵ (B) the section view of a fiber brush bioreactor loaded with *Caulobacter* cells, and (C) the overall bioremediation operation system, adapted from Shahbazi et al.¹⁵ Not to scale.

biofilms loses its stability in high concentrations of detergent, such as 0.1% (*w/v*) sodium dodecyl sulfate,⁹ which poses a restriction on the types of waste water that can be treated with such systems. On the other hand, detergent wash can be used as a convenient way to eradicate the biofilms when needed.

Besides the configuration of bioreactors, another direction worthwhile pursuing is to explore different biosupporting materials for optimal attachment

of JS4022/p723-6H. Although we have demonstrated that biofilms of JS4022/p723-6H formed on borosilicate surfaces are highly competent for sequestration of Cd(II) from water,¹⁰ glass is actually a poor support surface for *Caulobacter* spp. Dr. John Smit's group at the University of British Columbia has identified materials that could hold up to nine times as many *Caulobacter* cells as glass surfaces within an hour (personal communication). When necessary, a biosupporting unit may be

coated with one or multiple materials to achieve desirable immobilization results.

In summary, the construction of *Caulobacter* JS4022/p723-6H provides a practical solution toward the ultimate separation of dissolved toxic metals from the aqueous phase by microbes. Strain JS4022/p723-6H can effectively remove about half of the spiked cadmium from natural lake water either as free cells (Fig. 2) or as immobilized biofilms.¹⁰ Its tolerance to high acidity offers an

opportunity to regenerate the cells with acid treatments. Robust cells that are resistant to chelators can be obtained by supplying calcium to the growth media. Bioreactors with rich biosupporting surfaces, such as spiral sheet and fiber brush, are good candidates to host biofilms formed by *Caulobacter* strains. The technology we developed thus far is applicable to a variety of contaminated water sources, such as natural water bodies, drinking water, runoff and wastewaters low in detergent. To further improve the quality of the strain, additional engineering approaches may be attempted. For instance, longer histidine peptides may be incorporated to the cell surface for higher binding capacity per cell. Also, the gene encoding RsaA-6His fusion protein may be integrated into the host chromosome to avoid using antibiotics during cultivation of the cells or biofilms, and other types of affinity peptides may be incorporated instead for tailored specificity toward certain metals.

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