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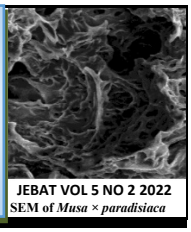

Entrapment of Mo-reducing Bacterium Increase its Resistance towards Mercury

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ABSTRACT

In ruminants, even trace amounts of molybdenum can be lethal. In areas with high pollution, molybdenum levels in soil and mine tailings can exceed 20,000 ppm. Bioremediation of molybdenum can be challenging when toxic mercury is also present. This research presents a novel approach using dialysis tubing and the molybdenum-reducing activity of *Enterobacter* sp. strain Dr. Y13 for molybdenum removal from aqueous solutions. Molybdenum blue (Mo-blue), produced during enzymatic reduction, is insoluble in dialysis tubing and this can be a twofold advantage as a method of removal and as a method to protect bacterial cells from heavy metal inhibition, especially mercury. In this experiment, we assess the toxicity-shielding effect of dialysis tubing for molybdenum reduction to Mo-blue by this bacterium in the presence of mercury. As the concentrations of mercury were increased, both free and immobilized cells were strongly inhibited. Modelling using the dissociation–one phase exponential decay model gave an IC_{50} value for the immobilized form of 0.1107 mg/L (95% confidence interval from 0.073 to 0.217 while the IC_{50} value for the free cell system was 0.023 mg/L (95% C.I. from 0.019 to 0.028). Since the confidence interval for the IC_{50} values did not overlap, the immobilized system gave better protection from mercury than the free cell system. Toxicity to free cells was higher than toxicity to cells trapped in dialysis tubes, suggesting that trapping Mo-reducing cells may be an effective strategy for bioremediation of water or wastewater contaminated with multiple heavy metals.

INTRODUCTION

Metal toxicity is typically attributed to metal ions' strong binding to the sulfhydryl (-SH) groups of enzymes involved in vital microbial metabolic processes. Metals can interfere with pollutant biodegradation and remediation in two ways: by interacting with enzymes specifically involved in the process (such pollutant-specific oxygenases or metal-reducing enzyme), or by interacting with enzymes involved in general metabolism. In both cases, the ionic form of the metal is responsible for the inhibition. This indicates that ionic species concentration, and not only total or even total soluble metal concentration, is crucial in determining metal toxicity (which may include metal-organic complexes that are not capable of binding to enzymes). The relevant metal concentration is thus that which may bind to enzymes and so inhibit microbial action. Despite the significance of the idea of bioavailable metal, it is challenging to evaluate bioavailable metal since it changes with both environment and organism [1–3].

Several methods exist for completing biodegradation tasks in the presence of heavy metal inhibitors. If a main bacterial degrader is already present, adding a metal-resistant bacterium can speed up the breakdown process. One investigation using soil microcosms with cadmium-contaminated soil spiked with a cadmium-resistant *Pseudomonas* sp. H1 strain that accumulates

cadmium intracellularly and is a 2,4-D-degrading bacterium. The findings demonstrate that inoculating with metal-resistant bacteria that decrease bioavailable metal concentrations through sequestration would promote greater biodegradation in the presence of a hazardous metal [4].

Metal bioavailability and mobility can be decreased by adding treatment additives to metal-contaminated areas, such as calcium carbonate, phosphate, cement, manganese oxide, and magnesium hydroxide [5]. Including clay minerals is still another option. Clay minerals have been used to lower metal bioavailability and toxicity. There was a significant decrease in cadmium's toxicity to yeasts, bacteria, and an actinomycete when kaolinite (1-20%) or montmorillonite (1-5%) was added to an agar medium containing the metal [6]. Similarly, Kamel (1986) found that the toxicity of 150 mg total cadmium/L to *Streptomyces bottropensis* may be mitigated by adding 3 percent bentonite and vermiculite to the solution. Kaolinite, like the other clays, decreased cadmium toxicity, although at a higher concentration (6 percent vs. 3 percent) and with less protection [7]. The use of immobilized bacteria to combat metal toxicity [8–12] is another avenue.

There are three different types of mercury: inorganic mercury, metallic elemental mercury and organic mercury (methylmercury). These types have varying degrees of toxicity and effects on human organs like the gut, brain, and immune system, as well as the eyes, skin, and lungs. Although traces of mercury can be found in the earth's crust, its release into the environment has been substantially exacerbated by human activities such as coal-fired power production, industrial operations, waste incineration, domestic coal burning and mercury (and gold and other metals) mining. Methylmercury, which bacteria may turn mercury into, bioaccumulates in fish and shellfish and biomagnetizes up the food chain, with large predatory fish having higher quantities than smaller fish. There are several routes of exposure to mercury, including ingestion of contaminated fish and shellfish and inhalation of elemental mercury fumes in industrial settings. The mercury remains even after cooking [13–19].

Pollution levels of molybdenum and other heavy metals have been measured across the world. Hundreds of parts per million (ppm) of molybdenum have been found in Tokyo Bay, one example of Japanese marine pollution. While humans are not directly exposed to molybdenum's toxicity, ruminants such as cows are very susceptible; scouring has been documented in places contaminated with molybdenum at levels as low as a few parts per million. Large tracts of grassland in Tyrol, Austria have been affected by molybdenum contamination, with concentrations as high as 200 parts per trillion. The first known instance of molybdenum bioremediation utilizing microorganisms and plants occurred in this region. As a byproduct of copper mining, molybdenum in the form of molybdenite is extracted in Malaysia [14–22]. However, there have been several incidents of contamination due to the inadvertent leaking of pipes carrying the metal. There has been a lot of research on the possibility of using microorganisms to detoxify metals. Metals may be removed in a few ways. Enzymatic reduction of metal into a less hazardous precipitable state is one of them. One particularly eye-catching instance is the reduction of molybdenum to molybdenum blue (Mo-blue), a precipitable material with a stunning blue color [3,20–29]. It has been known for at least the last century that some microorganisms can convert molybdate into Mo-blue. Despite this, metal ions, especially mercury, are powerful inhibitors of bioremediation, as is the case with many xenobiotics. As the dialysis tube approach may shield the bioreduction process from heavy metals, it is an appealing bioremoval technology [37–41]. This work reports for the first time the possible application of this approach in safeguarding molybdenum removal by a bacterium in the presence of mercury.

MATERIALS AND METHODS

Bacterial growth and maintenance of the Mo-reducing *Enterobacter* sp. strain Dr. Y13 was maintained on a solid agar of low phosphate (2.9 mM phosphate) medium (pH 7.0) consisting of (w/v%) sucrose (1%), $(NH_4)_2SO_4$ (0.3%), $MgSO_4 \cdot 7H_2O$ (0.05%), NaCl (0.5%), yeast extract (0.05%), $Na_2MoO_4 \cdot 2H_2O$ (0.726 %) and Na_2HPO_4 (0.073%). Sucrose needs to be autoclaved independently. Similar conditions to those used for solid-phase growth are employed for liquid-phase growth; however, a high phosphate medium (containing 100 mM phosphate) is used (HPM). It is simply the phosphate concentration that varies between the high and low phosphate medium. *Enterobacter* sp. strain Dr. Y13 was cultured in 5 L of HPM in two 5 L conical flasks at 30 °C with an orbital shaker for 48 hours to facilitate a large-scale cultivation (100 rpm, Kubota). Molybdenum blue formation in the medium was evaluated at 865 nm. The specific extinction coefficient is 16.7 $mM.^{-1}.cm^{-1}$ at 865 nm [30,31].

Cells were harvested by centrifugation at 15,000 g for 10 minutes and the pellet was resuspended in the low phosphate solution to an absorbance at 600 nm of approximately 1.00. A 10 mL bacterial suspension was cultured in 100 mL of sterile LPM medium (pH 7.0) with varying concentrations of mercury (AAS Merck 1000 mg/L stock standard solution) and incubated statically at 30°C in dialysis tubing pre-heated for 10 minutes. 1 mL aliquots were taken at regular intervals, centrifuged at 15,000 ×g for 15 minutes, and absorbance was measured at 865 nm. Three trials were conducted.

Modelling of mercury inhibition

The inhibitory effects of mercury to Mo-reduction by the bacterium were modelled according to the dissociation–one phase exponential decay. Fitting of the curve was carried out using the CurveExpert software (v1.6).

RESULTS AND DISCUSSION

The presence of reducing agents may be detected with great precision using Mo-blue. Molybdate (and molybdophosphate) may be reduced to Mo-blue by a wide variety of chemical and inorganic reducing agents. Therefore, it is unclear whether the reduction is enzymatic or the result of bioreductants generated by the cells. It's also possible that both processes are occurring concurrently, adding to the total Mo-reducing activity. The use of dialysis tubing has been demonstrated as a potential way of differentiation in this context [11]. The molybdenum blue product's colloidal feature is used in the molybdenum removal procedure from water.

Mercury showed strong inhibition towards both free and entrapped cells with a significantly higher inhibition ($p<0.05$) in the free cells system (**Fig. 1**). As the concentrations of mercury were increased, both free and immobilized cells were strongly inhibited. Modelling using the dissociation–one phase exponential decay (**Fig. 2**) model gave an IC_{50} value for the immobilized form of 0.1107 mg/L (95% confidence interval from 0.073 to 0.217 while the IC_{50} value for the free cell system was 0.023 mg/L (95% C.I. from 0.019 to 0.028). Since the confidence interval for the IC_{50} values did not overlap, the immobilized

system gave better protection from mercury than the free cell system.

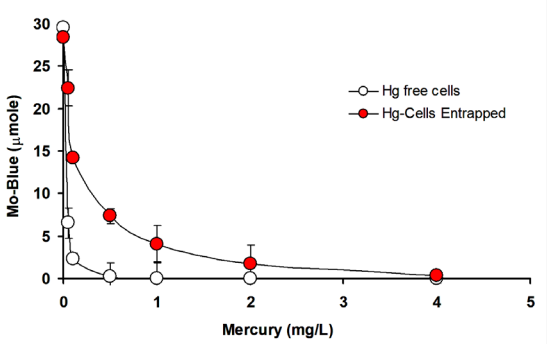

Fig. 1. The effect of increasing concentration of mercury to molybdenum blue reduction by *Enterobacter* sp. strain Dr. Y13 in the free-(○) and immobilized (●) systems. Data indicate the mean standard deviation of triplicates.

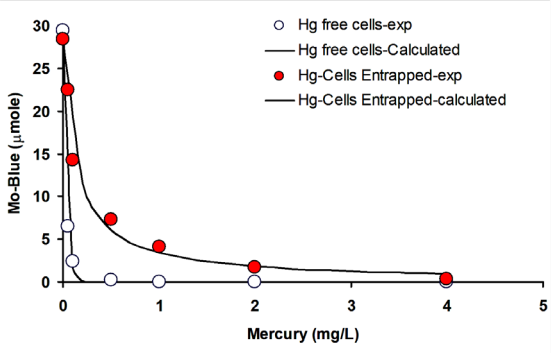

Fig. 2. Modelling the effect of increasing concentration of mercury to molybdenum blue reduction by *Enterobacter* sp. strain Dr. Y13 in the free-(○) and immobilized (●) systems using the dissociation−one phase exponential decay (solid curve). Data indicate the mean standard deviation of triplicates.

The attenuated effects of heavy metals toxicity to enzymatic molybdenum reduction are most likely the result of a combination of factors. These factors include diffusion retardation caused by the dialysis tubing, adsorption of heavy metals to the cellulose tubings, and adsorption to the negatively charged precipitated Mo-blue mass on the cells' surface. Increasing the stability and effectiveness of an enzyme or cell can be accomplished by immobilizing it or capturing it. Resistance to heavy metal is a desirable feature. To this day, the vast majority of bacteria that reduce molybdenum are sensitive to concentrations of mercury that are less than 1 mg/L. This indicates that mercury is toxic to the reduction process, which is a regular occurrence in many bioremediation activities [32–39]. The resistance to mercury in its encapsulated form can be improved. It is possible that in the future, many potential immobilization or entrapment matrices, such as alginate, chitosan, and polyacrylamide, will be investigated in order to evaluate the resistance of these matrices to heavy metals and the efficiency with which they reduce these metals.

CONCLUSION

We conclude that the dialysis tubing approach has the potential as a bioremediation tool, in particular for molybdenum in wastewater effluents and pretreatment systems in the presence of toxic mercury. The removal rate, indicating an effective removal system, would be useful for businesses whose waste contains high concentrations of molybdenum, such as the pigment and dye industries and molybdenum mine tailing effluents co-contaminated with mercury. Future research might focus on the synergistic protective effects of dialysis tubing and precipitated mass on the cell surface in response to mercury exposure.

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