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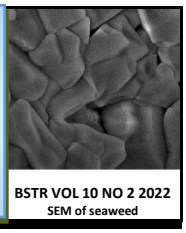

Immobilization of *Bacillus* sp. Strain Neni-8 in Dialysis Tubing Reduced Copper Toxicity to the Molybdenum Reduction Process

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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 copper is also present. This research presents a novel approach using dialysis tubing and the molybdenum-reducing activity of *Bacillus* sp. strain Neni-8 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 copper. 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 copper. As the concentrations of copper 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 copper 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 the bioremediation of water or wastewater contaminated with multiple heavy metals.

INTRODUCTION

High quantities of copper, a trace metal (needed by humans in levels of 1 to 100 mg per day), are located in the brain, liver, and kidney. However, more than half of the copper in the body is found in bone and muscle due to its large sizes. Ceruloplasmin is a liver protein that transfers copper linked to ceruloplasmin to the rest of the body. About half of the copper in the body is eliminated in the bile, with the other half leaving the body through various gastrointestinal secretions. As a result, copper homeostasis is mostly controlled by the digestive system. Numerous proteins rely on copper as a catalytic cofactor in redox chemistry, yet an excess of free copper ions can be harmful to the body [1–5]. The quantity of copper in a cell is controlled by a finely tuned balancing act between copper ion absorption and outflow. Oxidative stress, DNA damage, and a decrease in cell proliferation are all brought on by copper overload. Copper sulfate is harmful if more than 1 gram is ingested. When a metabolic disorder is inherited, the resulting copper toxicosis is called primary copper toxicosis, but when the disorder is the consequence of excessive copper consumption, increased copper absorption, or decreased copper excretion, the disorder is called secondary copper toxicosis. Consuming acidic meals cooked on uncoated copper cookware or being exposed to excess copper in drinking water or other environmental sources can lead to copperiedus (copper toxicity) [1–11].

Consumption of polluted water, use of copper salt-containing topical creams for burn treatments, preparation of acidic foods in uncoated copper cookware, and attempted suicide

(the fatal amount of swallowed copper is 0.015 grams) are common causes of copper poisoning (10 to 20 g). In many countries, copper sulfate may be purchased without a prescription. It has several practical uses, including as a pesticide, in the tanning industry, and for the production of leather and homemade glue. Accidental poisonings from copper sulfate crystals are common because youngsters are drawn to their brilliant blue hue. [6] An abnormality in the gene that codes for the copper-ATPase enzyme results in Wilson disease, an autosomal recessive ailment characterized by a buildup of copper in the cells [1–11]. Eighty-five percent to ninety-five percent of the copper in the blood is attached to ceruloplasmin, while the remaining five percent or so is "free," loosely connected to albumin and other tiny molecules [12,13].

Molybdenum and other heavy metal pollution levels have been assessed on a global scale. One example of Japanese marine pollution is the discovery of hundreds of parts per billion (ppb) of molybdenum in Tokyo Bay. It has been proven that ruminants, such as cows, can experience scouring in areas contaminated with molybdenum at levels as low as a few parts per million, despite the fact that people are not directly exposed to the toxicity of molybdenum. Grassland in the Tyrol region of Austria has been contaminated with molybdenum at levels of up to hundreds of parts per million. This is the site of the first documented case of molybdenum bioremediation by the use of microbes and plants [14–22].

Molybdenite in Nigeria can only be found in Plateau state, Nigeria, specifically in Kigom, Jos. There has been a lot of research on the possibilities of employing microbes to detoxify metals. There are several methods for extracting metals. One of them is the enzymatic transformation of metals into precipitable forms in which they pose less risk. Reducing the toxicity of soluble molybdenum can result in the formation of molybdenum blue (Mo-blue), a precipitable substance with a beautiful blue color [23–33].

Despite this, metal ions, especially copper, are a powerful inhibitors of bioremediation, as is the case with many xenobiotics [34–36]. 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 copper.

MATERIALS AND METHODS

Bacterial growth and maintenance of the Mo-reducing *Bacillus* sp. strain Neni-8 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. *Bacillus* sp. strain Neni-8 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 [31,42].

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 copper (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 copper inhibition

The inhibitory effects of copper 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

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 [23,43,44].

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 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 [45].

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 [46]. 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 [47]. 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 [48].

The use of immobilized bacteria to combat metal toxicity [37–41] is another avenue. 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 [38]. The molybdenum blue product's colloidal feature is used in the molybdenum removal procedure from water.

Copper 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 copper 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 copper than the free cell system.

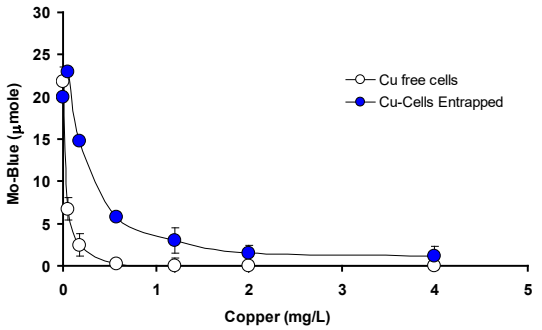

Fig. 1. The effect of increasing concentration of copper to molybdenum blue reduction by *Bacillus* sp. strain Neni-8 in the free-(○) and immobilized (●) systems. Data indicates mean standard deviation of triplicates.

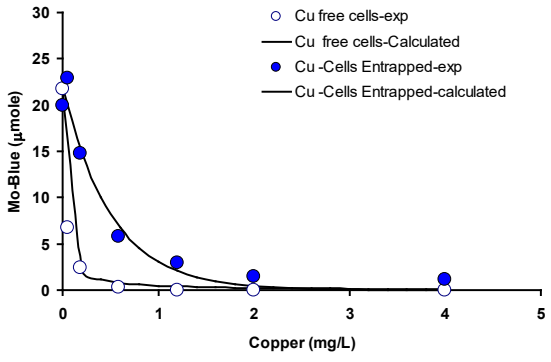

Fig. 2. Modelling the effect of increasing concentration of copper to molybdenum blue reduction by *Bacillus* sp. strain Neni-8 in the in the free-(○) and immobilized (●) systems using the dissociation–one phase exponential decay (solid curve). Data indicates mean standard deviation of triplicates.

The attenuated effects of heavy metals toxicity to enzymatic molybdenum reduction are likely due to a combination of factors, including diffusion retardation 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. Immobilizing or trapping an enzyme or cell can increase its stability and efficiency. Heavy metal resistance is a nice bonus.

Yet, the majority of Mo-reducing bacteria identified are sensitive to copper concentrations of less than 1 mg/L, indicating toxicity of copper to the reduction process, which is a common occurrence in many bioremediations works [49–56]. According to the findings of this study, the resistance to copper can be improved in the entrapped form. Alginate, chitosan, and polyacrylamide are only a few of the potential immobilization or entrapment matrices that may be explored in the future to evaluate their resistance to heavy metals and their effectiveness of reduction.

CONCLUSION

We come to the conclusion that the dialysis tubing method has the potential to be utilized as a bioremediation tool, in particular for the removal of molybdenum from wastewater effluents and pretreatment systems when poisonous copper is present. The removal rate, which 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 copper, would indicate an effective removal system. This would be beneficial for these types of industries. The combined protective effects of dialysis tubing and precipitated mass on the cell surface as a reaction to copper exposure might be the subject of study that will be conducted in the future.

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