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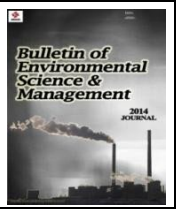

Short Communication: Entrapment of Mo-Reducing Bacterium Increase Its Resistance towards Heavy Metals

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ABBREVIATION
Mo-blue- molybdenum blue
$V_{MobluеMax}$-maximal rate of molybdenum blue production
K_{Mo} -half-maximal rate of reduction con

ABSTRACT

Molybdenum is highly toxic to ruminant at several ppm. Molybdenum pollution in soil and mine tailings could reach 20,000 ppm in the most contaminated region. Molybdenum is mined as a byproduct of copper in malaysia and occasional elevated levels of these heavy metals have been reported. Bioremediation of molybdenum in the presence of the toxic copper is a challenge. In this work a novel method of molybdenum removal from aqueous solution using the dialysis tubing method coupled with molybdenum-reducing activity of *serratia* sp. Strain dry5 is demonstrated. The enzymatic reduction of molybdenum is molybdenum blue, a colloid that does not pass through dialysis tubing. The calculated maximal rate of molybdenum blue production ($v_{mobluemax}$) was 0.264±0.034 mmole/mo-blue/hr and the concentration of molybdate resulting in the half-maximal rate of reduction (k_{mo}) was 21.78±3.89 mM molybdate indicating an efficient system with high tolerance towards molybdenum. Heavy metals exhibited significantly higher inhibition towards free cells compared to dialysis tubing entrapped cells. Hence the immobilization of mo-reducing cells by entrapment could be a viable bioremediation tool in aquatic bodies or effluent co-contaminated by other heavy metals.

Molybdenum is one of the heavy metals of which its pollution has been recorded globally [1]. For example, Japan has recorded evidence of molybdenum pollution in the sea in the Tokyo Bay where molybdenum level reaches hundreds of ppm [1]. Molybdenum is not toxic to human but very toxic to ruminants with scouring of cows have been reported to occur after grazing in areas polluted with several parts per million of molybdenum [2]. In Tyrol, Austria, molybdenum pollution has contaminated large pasture areas, reaching as high as 200 ppm. It is in this area that the first documented case of bioremediation of molybdenum was carried out using a combination of microbes and plants [3]. In Malaysia, molybdenum in the form of molybdenite is mined as a by-product of copper mining [4], and there have been reports of several cases of pollution caused by accidental leakage of pipe-carrying metal system [5]. The use of bacteria in metal removal has been extensively studied. One of the mechanisms of metal removal is via enzymatic reduction of metal into a less toxic precipitable form. Molybdenum reduction to molybdenum blue (Mo-blue) is a striking example with the reduced product exhibiting an intense blue precipitable mass [6]. Molybdate reduction by microbes to Mo-blue has been reported since the last one hundred years [6,-15]. Komori et al.[16] were the first to report on the bioremoval of chromate using dialysis tubing. The dialysis tubing method is an attractive bioremoval system as other immobilized systems tend to get clog or become impossible to remove the entangled mass of matrix, cells and reduced heavy metals precipitate. The potential use of this method in molybdenum removal is reported.

The growth and maintenance of *Serratia* sp. strain DRY5 was maintained on a solid agar of low phosphate (2.9 mM phosphate) media (pH 7.0) containing (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%) [11]. Sucrose was autoclaved separately. Growth in liquid media uses the same media as in the solid media above but 100 mM phosphate was used and this is called high phosphate media (HPM). The only difference between the high and low phosphate media is the phosphate concentration. For large-scale growth, *Serratia* sp. strain DRY5 was grown in 5 L of HPM in separate large conical flasks with a total 5 l capacity at 30 °C for 48 hours on an orbital shaker (100 rpm, Kubota). The production of molybdenum blue from the media was measured at 865 nm. The specific extinction coefficient is 16.7 $mM.^{-1}.cm^{-1}$ at 865 nm[10].

Cells were harvested by centrifugation at 15,000 g for 10 minutes and the pellet resuspended in low phosphate solution (pH 7.0) containing (w/v) $(NH_4)_2SO_4$ (0.3%), $MgSO_4 \cdot 7H_2O$ (0.05%), NaCl (0.5%), yeast extract (0.05%) and Na_2HPO_4 (0.05%)[11] to an absorbance at 600 nm of approximately 1.00. About 10 ml of this suspension was then placed in dialysis tubing previously boiled for ten minutes and immersed in sterile 100 ml of LPM media (pH 7.0) containing various concentrations of sodium molybdate and incubated statically at 30 °C. In the experiments on the effect of heavy metals, heavy metals such as silver (ii), cadmium (ii), copper (ii), mercury (ii) and lead (ii) were Atomic Absorption Spectrometry standard solutions from MERCK (Merck, Darmstadt, Germany). The heavy metals were added directly into the LPM to a final concentration of 10 mg/l. Aliquots (1 ml) of the media were periodically taken. The aliquots were centrifuged at 15,000 g for 15 minutes and the supernatant was then read at 865 nm. Experiments were carried out in triplicate. Values are means ± SE. All data were analyzed using Graphpad Prism version 3.0 available from www.graphpad.com. Comparison between groups was performed using a Student's t-test or a one-way analysis of variance with post hoc analysis by Tukey's test. $P < 0.05$ was considered statistically significant.

Mo-blue is a sensitive test for the presence of chemical-reducing agents [17]. This means that many reducing agents, organic or inorganic are capable of reducing molybdate (and molybdophosphate) to Mo-blue. Hence, it would be difficult to know whether the reduction is either enzymatic or due to bioreductants produced by the cells. Both processes could also contribute simultaneously to the overall Mo-reducing activity. It has been shown previously that the dialysis tubing method could be used as a distinguishing technique for this purpose [11]. The colloidal property of the molybdenum blue product is exploited in the removal process for molybdenum from aqueous environment.

All of the heavy metals tested showed strong inhibition towards both free and entrapped cells with a significantly higher inhibition ($p<0.05$) in the free cells system (Figure 1). The results (Figure 1) showed a general improvement of heavy metals toxicity after entrapment. Free cells are completely inhibited by 2 mg/l of heavy metals whilst entrapped cells could withstand at least twice the concentration of heavy metals. Copper is the most toxic with complete inhibition at 1 mg/l by free cells form and 2 mg/l by entrapped cells.

As the precipitable mass is highly negative in charge and dense, it is possible that the lessened effects of heavy metals toxicity to enzymatic reduction are probably due to several factors such as, diffusion retardation by the dialysis tubing, adsorption of heavy metals to the cellulose tubings, adsorption to the negatively-charged precipitated Mo-blue mass on the cells surface as well as the precipitated mass forming a barrier towards inhibition of Mo-blue production.

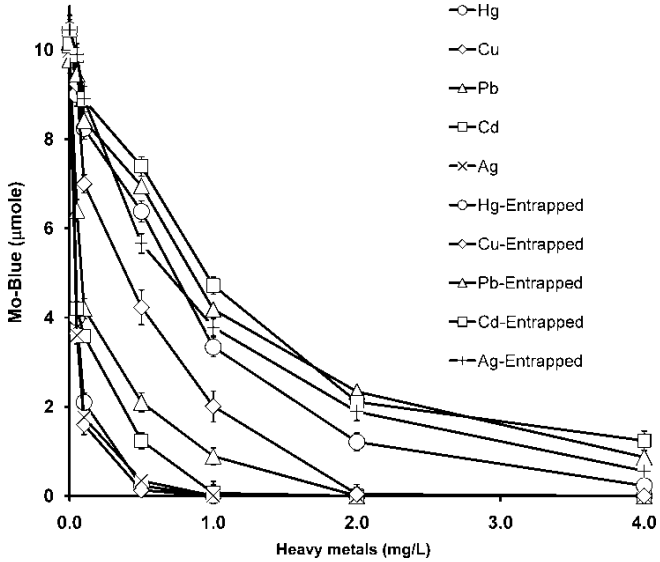

Figure 1. The effects of heavy metals on Mo-blue production in free cells and dialysis tubing system after a 24 hour static incubation at 30 °C. Data is mean ± standard error of the mean (n=3).

Generally, immobilization or entrapment is the method of choice for improving stability and efficiency of enzymes and cells [18-21]. An additional benefit is heavy metals resistance [22].

Majority of the Mo-reducing bacteria isolated to date is sensitive to copper at less than 1 mg/l [10-15]. In the entrapped form the resistance towards copper could be raised higher based on the results in this work. In the future, other form and matrix of immobilization or entrapment such as alginate, chitosan and polyacrylamide would be pursued to compare the resistant towards heavy metals and efficiency of reduction.

In conclusion, the dialysis tubing method could be exploited as a tool for bioremediation especially for molybdenum in waste water effluents or pretreatment system. The removal rate reflects an efficient removal system and would benefited industries that have high molybdenum content in their waste such as the pigment and dye industries and molybdenum mine tailing effluents. The protective combinatorial effects of the dialysis tubing and the precipitated mass on the cell surface to heavy metals toxicity would be the future works.

REFERENCES

[1] Davis, G K, Molybdenum, in *Metals and their compounds in the environment, occurrence, analysis and biological relevance*, edited by E Merian, (VCH Weinheim, New York) 1991, 1089–1100.

[2] Sas B, Secondary copper deficiency in cattle caused by molybdenum contamination of fodder, a case history, *Vet Hum Toxicol*, 3(1) (1989) 29–33.

[3] Neunhäuserer C, Berreck M & Insam H, Remediation of soils contaminated with molybdenum using soil amendments and phytoremediation, *Water Air Soil Poll*, 128 (2001) 85–96.

[4] Kosaka H & Wakita K, Some geologic features of the Mamut porphyry copper deposit, Sabah, *Malaysia Econ Geol*, 73(5) (1978) 618–627.

[5] Yong F S, Mamut Copper Mine - The untold story, minerals : underpinning yesterday's needs, in *Today's development and tomorrows's growth*, held on 22-24 June, 2000 (The National Seminar On The Malaysian Minerals Industry, Pacific Sutera Hotel Kota Kinabalu, Sabah) 2000.

[6] Campbell M A, Campbell A D & Villaret D B, Molybdate reduction by *Escherichia coli* K-12 and its chl mutants, *Proc Natl Acad Sci USA*, 82 (1985) 227–231.

[7] Capaldi A & Proskauer B, Beiträge zur Kenntnis der Säurebildung bei Typhusbacillen und Bacterium coli, *Z Hyg Infekt-Kr*, 23(1896) 452–474.

[8] Sugio T, Tsujita Y, Katagiri T, Inagaki K & Tano T, Reduction of Mo^{6+} with elemental sulfur by *Thiobacillus ferrooxidans*, *J Bacteriol*, 170(12) (1988) 5956–5959.

[9] Ghani B, Takai M, Hisham N Z, Kishimito N, Ismail M I A, Tano T & Sugio T, Isolation and characterization of a Mo^{6+}-reducing bacterium, *Appl Environ Microbiol*, 59 (1993) 1176–1180.

[10] Shukor M Y, Shamaan N A, Syed M A, Lee C H & Karim M I A, Characterization and quantification of molybdenum blue production in *Enterobacter cloacae* strain 48 using 12-molybdophosphate as the reference compound, *Asia Pac J Mol Biol Biotechnol*, 8(2) (2000) 167–172.

[11] Rahman M F A, Shukor M Y, Suhaili Z, Mustafa S, Shamaan N A & Syed M A, Reduction of Mo(VI) by the bacterium *Serratia* sp strain DRY5, *J Environ Biol*, 30(1) (2009) 65–72.

[12] Shukor M Y, Syed M A, Lee C H, Karim M I A & Shamaan N A, A method to distinguish between chemical and enzymatic reduction of molybdenum in *Enterobacter cloacae* strain 48, Malaysian J Biochem, 7 (2002) 71–72.

[13] Shukor M Y, Habib S H M, Rahman M F A, Jirangon H, Abdullah M P A, Shamaan N A & Syed M A, Hexavalent molybdenum reduction to molybdenum blue by *S marcescens* strain DrY6, *Appl Biochem Biotechnol*, 149(1) (2008) 33–43.

[14] Shukor M Y, Rahman M F, Suhaili Z, Shamaan N A & Syed M A, Hexavalent molybdenum reduction to mo-blue by *Acinetobacter calcoaceticus*, *Folia Microbiol*, 55(2) (2010) 137–143.

[15] Shukor M Y, Ahmad S A, Abdullah M P, Shamaan NA & Syed M A, Molybdate reduction by *Pseudomonas* sp strain DRY2, *J Appl Microbiol*, 108 (2010) 2050–2058.

[16] Komori K, Rivas A, Toda K & Ohtake H, A method for removal of toxic chromium using dialysis-sac cultures of a chromate-reducing strain of *Enterobacter cloacae, Appl Microbiol Biotechnol*, 33 (1990) 117–119.

[17] Killefer D H & Linz A, *Molybdenum compounds; their chemistry and technology* (Interscience Publisher, New York) 1952.

[18] Sarma S J, Pakshirajan K & Saamrat KBG, Pyrene biodegradation by free and immobilized cells of Mycobacterium frederiksbergense using a solvent encapsulated system, *Indian J Biotechnol*, 10(4) (2011) 496–501.

[19] West T P, Effect of carbon source on polysaccharide production by alginate-entrapped *Aureobasidium pullulans* ATCC 42023 cells, *J Basic Microbiol*, 51(6) (2011) 673–677.

[20] Stanescu M D, Sanislav A, Ivanov R V, Hirtopeanu A, Lozinsky V I, Immobilized laccase on a new cryogel carrier and kinetics of two anthraquinone derivatives oxidation, *Appl Biochem Biotechnol*, 165(7-8) (2011) 1789–1798.

[21] Sharma V K & Singh R P, Organic matrix based slow release fertilizer enhances plant growth, nitrate assimilation and seed yield of Indian mustard (Brassica juncea L.), *J Environ Biol*, 32(5) (2011) 619–624.

[22] Banerjee M, Mishra S & Chatterjee J., Scavenging of nickel and chromium toxicity in *Aulosira fertilissima* by immobilization: Effect on nitrogen assimilating enzymes, *Electron J Biotechnol*, 7(3) (2004) doi: 10.2225/vol7-issue3-fulltext-9.