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Short communication

A Microplate Format for Characterizing the Growth of Molybdenum-reducing Bacteria

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ABSTRACT

Molybdenum is toxic to ruminants and the process of spermatogenesis in catfish and mice. The bioremediation of heavy metals including molybdenum is being intensely studied. More efficient reducers are being isolated. An advantage of microbial molybdate reduction to molybdenum blue is the intense blue product absorbs wavelength strongly in the far red region whilst cells without the addition of molybdenum showed little absorption in this region. This means that no sample treatments such as centrifugation are needed and the readings can be taken straight away. In this work we developed a microplate or a microtiter plate assay for monitoring molybdenum blue production from a bacterium. The molybdenum blue produced when measured from a microplate gave about 2 times less absorbance intensity compared to measurement using a normal cuvette, but is adequate for characterization works as the absorbance obtained was 0.886 compared to 1.709 for measurement using cuvette. The development of microplate format for monitoring microbial reduction to molybdenum blue is simple to perform and would ensure works on the characterizations of novel molybdenum reducers can potentially be carried out in a few days instead of a few weeks.

INTRODUCTION

Molybdenum is a heavy metal that exhibit toxicity to humans. However, it is very toxic to ruminants. It has been discovered that levels as low as several parts per million can cause scouring in ruminants and in certain cases this could lead to death [1,2]. Furthermore, works have also shown that molybdenum could inhibit the process of spermatogenesis in animals such as catfish and mice at low concentrations [3–5]. Molybdenum toxicity has also been demonstrated in small organisms such as fries of chinook and coho salmons and the amphipod *Allorchestes compressa* [6,7]. As molybdenum pollution has been recorded at an ever increasing level globally, there is the need to find ways to remove molybdenum from soils and aquatic bodies. For the last few decades, researchers have focused on bioremediation as a low cost method and an environmental friendly way to solve this issue [8]. The use of microorganisms to remediate heavy metals such as chromium and mercury is well known and has been reported frequently in the literature. In the case of molybdenum not many works have been done.

Currently, the majority of the Mo-reducing bacteria have been isolated from Malaysian and Pakistanis soils [9–23,23–29]. As screening and characterizing works forms a major part of the process in finding novel and better molybdenum reducers, ways to sped up these processes could benefit bioremediation works. An example of such an effort is the development of a simple microtiter plate assay for monitoring chromium reduction in bacterium developed by Han et al [30]. In this work we developed a microplate format for monitoring molybdenum blue production from a bacterium and compared its efficacy to the normal measurement using cuvette. We discovered that the readings obtained from the microplate format using resting cells gave adequate signal for routine screening and characterization works.

MATERIALS AND METHODS

Growth and maintenance of Mo-reducing bacterium

Serratia sp. strain DRY5 was previously isolated from a contaminated soil [9]. 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%), $Na_2MoO_4 \cdot 2H_2O$ (0.483 % or 20 mM) and Na_2HPO_4 (0.073% or 5 mM) [19]. 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 was the phosphate concentration. For large-scale growth, *Serratia* sp. strain DRY5 was grown in 1 L of HPM at 30 ^oC 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 [31].

Preparation of resting cells

Cells were harvested by centrifugation at 15,000 g for 10 minutes and the pellet washed several times and resuspended in 20 mls of low phosphate media (LPM) minus sucrose to an absorbance at 600 nm of approximately 1.00. Then 180 µL was sterically pipetted into each well of a sterile microplate. 20 µL of sterile sucrose from a stock solution was then added to each well to initiate molybdenum blue production. A sterile sealing tape that allows gas exchange (Corning® microplate) was used for sealing the tape. The microplate was incubated at 30 ^oC. At defined times; absorbance at 750 nm was read in a BioRad (Richmond, CA) Microtiter Plate reader (Model No. 680). Similar experiment was carried out for measurement of molybdenum blue in a cuvette. This was carried out in sealed sterile test tubes with the same conditions as the microplate format with the exception where the total volume of the single cuvette measurement experiment was carried out for a 10 ml incubation mixture.

RESULT AND DISCUSSION

The advantage of microbial molybdate reduction to molybdenum blue is the intense blue solution absorbs wavelength strongly in the far red region whilst resting cells without the addition of molybdenum showed little absorption in this region (**Fig**. 1). This means that no sample treatments such as centrifugation are needed and the readings can be taken straight away. The signal to control ratio was also very high even at 600 nm indicating measurements can be made using simple microplate reader such as the popular Biorad 680 with a 750 nm filter, the maximum wavelength available for this model. At this wavelength, the reduction in absorbance compared to measurement at 865 nm is approximately 30% less (**Table** 1).

Table 1. Comparison between microplate format and single spectrophotometric measurements of bacterial molybdenum blue produced by *Serratia* sp. strain DRY5.

Incubation (hr)	Abs 750 nm (Microtiter plate)	Abs 865 nm (Spectrophotometer)	Control (Microtiter plate, 750 nm)	Control (Spectrophotometer, 865 nm)
0	0.022±0.013	0.045±0.008	0.010±0.005	0.023±0.008
6	0.349±0.032	0.831±0.025	0.024±0.004	0.069±0.016
12	0.886±0.035	1.709±0.029	0.036±0.016	0.088±0.013

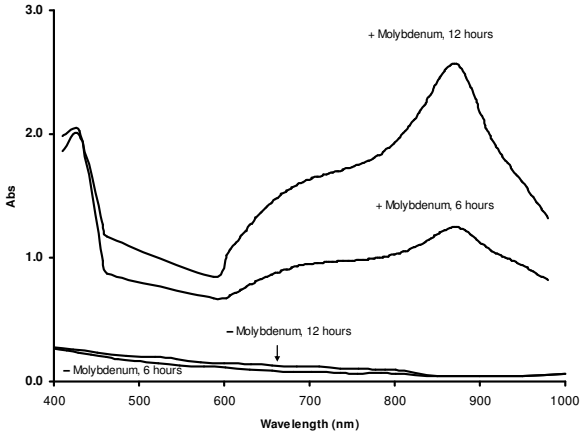

Fig. 1. Scanning spectra of resting cells supplemented with 20 mM molybdenum after 6 and 12 hours of incubation in a cuvette in comparison with non-supplemented control.

The pathlength of the light traversing the microplate when filled to 0.2 ml is only about 0.5 cm compared to 1.0 cm for a single cuvette spectrophotometer and hence the absorbance using a microplate is about 2 times less than when using a cuvette. However, the advantage of the microplate format with 96 wells easily surpassed single measurement in a cuvette. This would allow at least 30 data points to be obtained for studying characteristics of reduction such as the effects of phosphate, molybdate and pH on reduction including triplicates and control. Development of high-throughput method of characterizing biological activity would simultaneously enhance the sensitivity of the method, reduce required effort and improve the reproducibility of the data obtained [28,32].

In conclusion, the developed microplate format for monitoring microbial reduction to molybdenum blue is simple to perform, produce adequate quantification of the product compared to control, would allow multiple characterizations to be carried out in a single plate and ensure works on the characterizations of novel molybdenum reducers can be potentially be carried out in a few days instead of a few weeks.

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REFERENCES

[1]. Bremner I. The toxicity of cadmium, zinc and molybdenum and their effects on copper metabolism. Proc Nutr Soc. 1979;38(2):235–42.
[2]. Bone PA. Copper deficiency, molybdenum toxicity and copper toxicity: Where are we now? Cattle Pract. 2010;18(2):73–5.
[3]. Bi C-M, Zhang Y-L, Liu F-J, Zhou T-Z, Yang Z-J, Gao S-Y, et al. The effect of molybdenum on the in vitro development of mouse preimplantation embryos. Syst Biol Reprod Med. 2013;59(2):69–73.
[4]. Yamaguchi S, Miura C, Ito A, Agusa T, Iwata H, Tanabe S, et al. Effects of lead, molybdenum, rubidium, arsenic and organochlorines on spermatogenesis in fish: Monitoring at Mekong Delta area and in vitro experiment. Aquat Toxicol. 2007;83(1):43–51.
[5]. Zhai X-W, Zhang Y-L, Qi Q, Bai Y, Chen X-L, Jin L-J, et al. Effects of molybdenum on sperm quality and testis oxidative stress. Syst Biol Reprod Med. 2013;59(5):251–5.

[6]. Ahsanullah M. Acute toxicity of chromium, mercury, molybdenum and nickel to the amphipod *Allorchestes compressa*. Aust J Mar Freshw Res. 1982;33(3):465–74.
[7]. Hamilton SJ, Buhl KJ. Acute toxicity of boron, molybdenum, and selenium to fry of chinook salmon and coho salmon. Arch Environ Contam Toxicol. 1990;19(3):366–73.
[8]. Neunhäuserer C, Berreck M, Insam H. Remediation of soils contaminated with molybdenum using soil amendments and phytoremediation. 2001;128(1-2):85–96.
[9]. Rahman MFA, Shukor MY, Suhaili Z, Mustafa S, Shamaan NA, Syed MA. Reduction of Mo(VI) by the bacterium *Serratia* sp. strain DRY5. J Environ Biol. 2009;30(1):65–72.
[10]. Abo-Shakeer LKA, Ahmad SA, Shukor MY, Shamaan NA, Syed MA. Isolation and characterization of a molybdenum-reducing *Bacillus pumilus* strain lbna. J Environ Microbiol Toxicol. 2013;1(1):9–14.
[11]. Ahmad SA, Halmi MIE, Shukor MY, Shamaan NA, Syed MA. Effect of metal ions on the molybdenum-reducing activity of *S. marcescens* strain DrY6. J Environ Microbiol Toxicol. 2013;1(1):5–8.
[12]. Ahmad SA, Shukor MY, Shamaan NA, Mac Cormack WP, Syed MA. Molybdate reduction to molybdenum blue by an antarctic bacterium. BioMed Res Int. 2013;2013.
[13]. Halmi MIE, Wasoh H, Sukor S, Ahmad SA, Yusof MT, Shukor MY. Bioremoval of molybdenum from aqueous solution. Int J Agric Biol. 2014;16(4):848–50.
[14]. Lim HK, Syed MA, Shukor MY. Reduction of molybdate to molybdenum blue by *Klebsiella* sp. strain hkeem. J Basic Microbiol. 2012;52(3):296–305.
[15]. Othman AR, Bakar NA, Halmi MIE, Johari WLW, Ahmad SA, Jirangon H, et al. Kinetics of molybdenum reduction to molybdenum blue by *Bacillus* sp. strain A.rzi. BioMed Res Int. 2013;2013.
[16]. Rahman MA, Ahmad SA, Salvam S, Halmi MIE, Yusof MT, Shukor MY, et al. Dialysis tubing experiment showed that molybdenum reduction in *S. marcescens* strain DrY6 is mediated by enzymatic action. J Environ Bioremed Toxicol. 2013;1(1):25–7.
[17]. Shukor MY, Ahmad SA, Nadzir MMM, Abdullah MP, Shamaan NA, Syed MA. Molybdate reduction by *Pseudomonas* sp. strain DRY2. J Appl Microbiol. 2010;108(6):2050–8.
[18]. Shukor MY, Dahalan FA, Jusoh AZ, Shamaan NA, Syed, M.A. Characterization of diesel-degrading enzymes from *Acinetobacter* sp. strain DRY12. Bioremediation Sci Technol Res. 2013;1(1):15–8.
[19]. Shukor MY, Habib SHM, Rahman MFA, Jirangon H, Abdullah MPA, Shamaan NA, et al. Hexavalent molybdenum reduction to molybdenum blue by *S. marcescens* strain Dr. Y6. Appl Biochem Biotechnol. 2008;149(1):33–43.
[20]. Shukor MY, Halmi MIE, Rahman MFA, Shamaan NA, Syed MA. Molybdenum reduction to molybdenum blue in *Serratia* sp. strain DRY5 is catalyzed by a novel molybdenum-reducing enzyme. BioMed Res Int. 2014;2014.
[21]. Shukor MY, Rahman MF, Shamaan NA, Syed MS. Reduction of molybdate to molybdenum blue by *Enterobacter* sp. strain Dr.Y13. J Basic Microbiol. 2009;49(SUPPL. 1):S43–54.
[22]. Shukor MY, Rahman MF, Suhaili Z, Shamaan NA, Syed MA. Bacterial reduction of hexavalent molybdenum to molybdenum blue. World J Microbiol Biotechnol. 2009;25(7):1225–34.
[23]. Shukor MY, Rahman MF, Suhaili Z, Shamaan NA, Syed MA. Hexavalent molybdenum reduction to Mo-blue by *Acinetobacter calcoaceticus*. Folia Microbiol (Praha). 2010;55(2):137–43.
[24]. Shukor MY, Shamaan NA, Syed MA, Lee CH, Karim MIA. 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. 2000;8(2):167–72.
[25]. Shukor MY, Syed MA, Lee CH, Karim MIA, Shamaan NA. A method to distinguish between chemical and enzymatic reduction of molybdenum in *Enterobacter cloacae* strain 48. Malays J Biochem. 2002;7:71–2.
[26]. Shukor Y, Adam H, Ithnin K, Yunus I, Shamaan NA, Syed A. Molybdate reduction to molybdenum blue in microbe proceeds via a phosphomolybdate intermediate. J Biol Sci. 2007;7(8):1448–52.
[27]. Shukor Y, Shamsuddin B, Mohamad O, Ithnin K, Shamaan NA, Syed MA. A method to study the effects of chemical and biological reduction of molybdate to molybdenum blue in bacteria. Pak J Biol Sci. 2008;11(4):672–5.
[28]. Wahab SMA, Gunasekaran B, Shaharuddin NA, Johari WLW, Halmi MIE, Said NAM, et al. A novel method for the determination of mercury in herbal preparation using an inhibitive assay based on the protease papain. J Environ Microbiol Toxicol. 2013;1(1):1–4.
[29]. Yunus SM, Hamim HM, Anas OM, Aripin SN, Arif SM. Mo (VI) reduction to molybdenum blue by *Serratia marcescens* strain Dr. Y9. Pol J Microbiol. 2009;58(2):141–7.
[30]. Han R, Geller JT, Yang L, Brodie EL, Larsen JT, Beller HR. Physiological and transcriptional studies of Cr(VI) reduction under aerobic and denitrifying conditions by an aquifer-derived pseudomonad. Environ Sci Technol. 2010;44(19):7491–7.
[31]. Shukor MY, Lee CH, Omar I, Karim MIA, Syed MA, Shamaan NA. Isolation and characterization of a molybdenum-reducing enzyme in *Enterobacter cloacae* strain 48. Pertanika J Sci Technol. 2003;11(2):261–72.
[32]. Iyamu EW, Asakura T, Woods GM. A colorimetric microplate assay method for high-throughput analysis of arginase activity in vitro. Anal Biochem. 2008;383(2):332–4.