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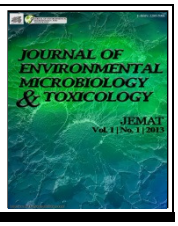

Short communication

Effect of Metal Ions on the Molybdenum-Reducing Activity of *S. marcescens* strain DrY6

Shukor M.Y.[1*], Shamaan N.A.[2], and M.A. Syed[1]

[1]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia

[2]Faculty of Medicine and Health Sciences, Universiti Sains Islam Malaysia, 13th Floor, Menara B, Persiaran MPAJ, Jalan Pandan Utama, Pandan Indah, 55100 Kuala Lumpur, Malaysia.

Corresponding author: Yunus Shukor; Tel No: +603-89466693; Fax No: +603-89430913; mohdyunus@upm.edu.my

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Abstract

Microbial metal reduction of molybdenum to molybdenum blue can be used in the bioremediation of molybdenum contaminated environment. A molybdenum-reducing strain, *S. marcescens* strain Dr.Y6, was tested for its molybdenum-reduction capability in the presence of several metal ions such as Cr^{6+}, Fe^{3+}, Fe^{2+}, Zn^{2+}, Mg^{2+}, Co^{2+},Ni^{2+},Cd^{2+}, Ag^{+}, Mn^{2+}, Cu^{2+}, Hg^{2+}, $Pb^{2+}$and Sn^{2+}. Metal ions such as chromium, copper and mercury caused 88.4, 98.8 and 88.3% inhibition of the molybdenum-reducing activity, respectively, indicating the presence of a thiol group in the molybdenum-reducing enzyme of this bacterium. However, two ions, ferrous and stannous, markedly increased the activity of molybdenum-reducing activity in this bacterium in the control studies suggesting that these metal ions could produce false positive results and their use should be accompanied with proper control.

INTRODUCTION

The prevalence of toxic xenobiotics in various parts of the world such as azo dyes [1-3], heavy metals [4-12], detergents [13], acrylamide [14,15], diesel [16,17] and pesticides [18,19] is such that it is no longer a question of "where" but a question of "how much". Thus, researchers have begun to embark upon various methods to reduce the toxicity of xenobiotics and heavy metals. Bioremediation is a biotechnological approach towards removal of heavy metal. Numerous heavy metals are amenable to bioremediation [20]. One of an emerging global metal pollutant is molybdenum [21]. Microbial molybdenum reduction to molybdenum blue is a phenomenon that have been reported for more than one hundred years and is a potential bioremediation tool. According to Levine, [22] microbial molybdate reduction to molybdenum blue was first mentioned in 1896 by Capaldi and Proskauer [23]. Other reports on mirobial molybdate reduction were by Jan [24], Marchal and Gerard [25], and Bautista and Alexander [26]. However, detailed studies on this phenomenon is initiated in 1985 by Campbell *et al*. [27] in *E. coli* K12. Several years later, Sugio *et al.* [28] reported on the reduction of molybdate into molybdenum blue by *Thiobacillus ferrooxidans* strain AP19-3 without being aware of the works carried out by Campbell *et al*. [27]. The first local bacterium reported with molybdenum-reducing ability is *Enterobacter cloacae* strain 48 (EC 48)[29]. It was discovered that several other molybdenum-reducing bacteria exhibited unique molybdenum blue absorption spectrum [30]. A method has been developed to test whether the effects of activators or inhibitors to the molybdenum-reducing enzyme are genuine or false [7]. Recently, we have isolated and characterized two local molybdenum-reducing bacteria- *Serratia marcescens* strain DrY6 [31] and *Serratia* sp. [5]. More recently, a novel inhibitive assay for the heavy metal copper has been developed using the molybdenum-reducing enzyme system [32]. Ghani *et al*. [29] and Campbell *et al*. [27] have shown that metal ions play an important role in molybdenum reduction. The effect of metal ions was not studied in *S. marcescens* strain DrY6 . Hence, we report on the study of the effect of metal ions on molybdate reduction by *S. marcescens* strain Dr.Y6.

S. marcescens was maintained in low phosphate molybdate media (LPM) media (pH 7.0) containing (w/v%) sucrose (1%), $(NH_4)_2SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.5%), yeast extract (0.05%), $Na_2MoO_4.2H_2O$ (20 mM) and Na_2HPO_4 (5 mM). Sucrose was autoclaved separately [31]. Growth in liquid media uses the same media as in the solid media above. Molybdenum blue is produced in this media but not at high phosphate media (100 mM phosphate). *S. marcescens* strain

Dr.Y6 was grown and maintained on the above low phosphate liquid and solid media.

Molybdenum-reducing enzyme was assayed using molybdate as the electron acceptor and NADH as the electron donor as outlined by Shukor *et al*., 2008d. Briefly, laboratory-prepared ten to four phosphomolybdate or 10:4 ratio of phosphomolybdate was prepared arbitrarily as a 60 mM stock solution in deionized water. This was achieved by mixing 600 mM molybdate ($Na_2MoO_4.2H_2O$) with 240 mM phosphate ($Na_2HPO_4.2H_2O$). Adjustment of the phosphomolybdate solution to pH 5.0 was carried out using 1 M HCl. Into 1 ml of reaction mixture containing 15 mM (final concentration) laboratory-prepared electron acceptor substrate in 50 mM citrate-phosphate buffer pH 5.0 at room temperature, 100 ml of NADH (80 mM stock) was added to a final concentration of 8 mM. Fifty microlitres of crude preparation of the Mo-reducing enzyme was added to start the reaction. The increase in absorbance in one minute was read at 865 nm. One unit of Mo-reducing activity is defined as that amount of enzyme that produce 1 nmole molybdenum blue per minute at room temperature. The specific extinction coefficient at 865 nm for the product; molybdenum blue, was determined by means of a standard curve obtained using ascorbate-reduced 12-phoshomolybdate. The specific extinction coefficient at 865 nm is 16.7 $mM^{-1}.cm^{-1}$. An increase in absorbance at 865 nm of 1.00 unit absorbance per minute per mg protein would yield 60 nmole of 12-phoshomolybdate or 60 units of enzyme activity in a 1 ml assay mixture [33].

The following experiment was carried out at 4 ^oC unless stated otherwise. Cells were harvested through centrifugation at 10 000 g for 10 minutes. Cells were washed at least once with distilled water, resuspended and recentrifuged. The pellet was reconstituted with 10 ml of 50 mM Tris buffer pH 7.5 (Tris buffer prepared at 4 ^oC) containing 0.1 mM PMSF (phenylmethylsulphonyl fluoride. Cells were sonicated for 1 minute on an ice bath with 4 minutes cooling until a total sonication time of at least 20 minutes was achieved. The sonicated fraction was centrifuged at 10 000 g for 20 minutes and the supernatant consisting of the crude enzyme fraction was taken.

Metal ions such as Fe^{3+} ($FeCl_3.6H_2O$, BDH), Cr^{6+} ($K_2Cr_2O_7$, BDH), Fe^{2+} ($Fe(NH_4)_2(SO_4)_2.6H_2O$, BDH), Zn^{2+} ($ZnCl_2$, BDH), Mg^{2+} ($MgCl_2$, BDH) , Co^{2+} ($CoCl_2.6H_2O$, BDH), Ni^{2+} ($NiCl_2.6H_2O$, BDH), Cd^{2+} ($CdCl_2.H_2O$, SparkChem), Ag^+ ($AgNO_3$, JT Baker) , Mn^{2+} ($MnCl_2.4H_2O$, JT Baker), Cu^{2+}($CuSO_4.5H_2O$, JT Baker), Hg^{2+} ($HgCl_2$ JT Baker), Pb^{2+} ($PbCl_2$, JT Baker) , Sn^{2+}($SnCl_2.2H_2O$, BDH) were dissolved in 20 mM Tris.Cl buffer pH 7.0. Inhibitors and metal ions were preincubated with one hundred microlitres of enzyme in the reaction mixture at 4 ^oC for 10 minutes minus NADH. The incubation mixture was then warmed to room temperature and NADH added to start the reaction. Deionised water was added so that the total reaction mixture was 2.0 mL. As a control, 50 mL of acetone was added in the reaction mixture without inhibitors. The increase in absorbance at 865 nm was measured after a period of 5 minutes.

The results showed that chromium, copper and mercury caused 88.4, 98.8 and 88.3% inhibition of the molybdenum-reducing activity, respectively. The results indicate the presence of sulfhydryl group in the active site of the molybdenum-reducing enzyme. Two ions; ferrous and stannous, markedly increased the activity of molybdenum-reducing activity in this bacterium. The rest of the metal ions did not have an effect on the molybdenum-reducing activity from this bacterium (**Table** 1).

TABLE 1. EFFECT OF METAL IONS ON MOLYBDATE REDUCTION. DATA IS MEAN± STANDARD ERROR (N=3).

Metal ions (2 mm)	Molybdenum blue produced (nmole/min/mg)
CONTROL	20.10 ± 0.17
Cr^{6+}	2.34 ± 0.11
Fe^{3+}	21.72 ± 2.21
Fe^{2+}	51.20 ± 2.34
Zn^{2+}	21.13 ± 0.15
Mg^{2+}	19.53 ± 1.24
Co^{2+}	21.13 ± 0.97
Ni^{2+}	20.28 ± 0.47
Cd^{2+}	18.59 ± 1.93
Ag^+	22.11 ± 2.75
Mn^{2+}	19.43 ± 0.29
Cu^{2+}	0.25 ± 0.06
Hg^{2+}	3.37 ± 0.96
Pb^{2+}	19.24 ± 0.11
Sn^{2+}	48.14 ± 1.54

However, we discovered that ferrous and stannous ions could reduce molybdate to molybdenum blue in the control studies. The stimulatory effect of ferrous and stannous is suspected since both have been used as chemical reducing agents for the conversion of molybdate to molybdenum blue [29]. In addition, Yong *et al*. [34] discovered that ferrous irons were responsible for the reduction of molybdate to molybdenum blue in the acidic media of *T. ferrooxidans*. Even the construction of the molybdenum blue standard curve uses stannous ions as the chemical reductant [28,29].

Certain metal ions especially heavy metals are known inhibitors of metal-reducing enzyme and the target of inhibition has been suggested as the thiol group [34, 35]. Thus, screening for metal tolerant strains is important since sites containing metal contaminant usually contain other toxic heavy metals. The remediation of a target metal would depend upon microbial resistance towards other metal ions presence in the site. Chromium-reducing bacteria relies upon chromate reductase to convert the soluble chromium (vi) ions into the less toxic chromium (iii) ions and this enzyme is inhibited by heavy metal ions such as copper and mercury. In order to overcome this inhibition, heavy metals resistant chromate-reducing bacteria have been screened from environmental isolates and the results have been successful [36]. Based on this approach, future works would include screening for metal resistant molybdenum reducing bacteria.

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