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Kinetic Studies of the Partially Purified Molybdenum-reducing Enzyme from *Bacillus pumilus* strain Lbna

Abo-Shakeer, L.K.A.[1], Rahman, M.F.A.[1], Yakasai, H.[1], Bakar, N.A.[2], Othman, A.R.[3*], Syed, M.A.[1], Abdullah, N.[4], Shukor M.Y.[1]

[1]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia.
[2]Institute of Ocean and Earth Sciences, Universiti Malaya, 50603 Kuala Lumpur, Malaysia.
[3] Department of Chemical Engineering and Process, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia (UKM), 43600 Bangi, Selangor, Malaysia.
[4]Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia 43400 UPM Serdang Selangor Darul Ehsan, Malaysia.

*Corresponding author: Ahmad Razi Othman
Department of Chemical Engineering and Process,
Faculty of Engineering and Built Environment,
Universiti Kebangsaan Malaysia (UKM),
43600 Bangi, Selangor,
Malaysia.
Email: ahmadrazi@ukm.edu.my

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ABSTRACT

Bacterial based remediation of environmental toxicants is a promising innovative technology for molybdenum pollution. To date, the enzyme responsible for molybdate reduction to Mo-blue from bacteria show that the Michaelis-Menten constants varies by one order of magnitude. It is important that the constants from newer enzyme sources be characterized so that a comparison can be made. The aim of this study is to characterize kinetically the enzyme from a previously isolated Mo-reducing bacterium; *Bacillus pumilus* strain Lbna. The maximum activity of this enzyme occurred at pH 5.5 and in between 25 and 35 °C. The K_m and V_{max} of NADH were 6.646 mM and 0.057 unit/mg enzyme, while the K_m and V_{max} of LPPM were 3.399 mM and 0.106 unit/mg enzyme. The results showed that the enzyme activity for *Bacillus pumilus* strain Lbna were inhibited by all heavy metals used. Zinc, copper, silver, chromium, cadmium and mercury all caused more than 50% inhibition to the Mo-reducing enzyme activity with copper being the most potent with an almost complete inhibition of enzyme activity observed.

INTRODUCTION

Pure molybdenum is found as a silvery white metal harboring various oxidation states between 2 and 6. The most stable forms of molybdenum are Mo^{4+} and Mo^{6+} [1]. Molybdenum is found in aqueous environment as molybdate anions (MoO_4^{2-}), that can form polymolybdate compounds under acidic conditions [2]. Molybdenum toxicity varies according to the route of ingestion. The absorption rate following oral ingestion depends on the compounds solubility and the diet composition [3]. Similarly, the chemical form in which molybdenum exist greatly influenced the rate of its bioavailability and also depends on the animal species [1,4–13]. In ruminants, however, concentrations of 10 mg/kg of body weight molybdenum resulted in tissue copper depletion, potentiated by dietary sulfate [14,15]. High doses of molybdenum at greater than 100 mg/L show a negative effect (exhibited a complete lack of libido and sterility) together with accompanying alterations in the levels of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in mice testes [3]. In a similar report, [16] revealed a decrease in germ cells number and mature spermatocytes in the testes of rabbits fed with carrots having 39 mg Mo/kg dry weight, and the appearance of degenerated cells, large number of syncytial giant cells among the spermatogenic cells in the seminiferous tubules relative to control.

Bacterial based remediation of environmental toxicants is a promising innovative technology that attracted great interest in recent years [17–25]. Mechanisms such as bioreduction, bioprecipitation, bioaccumulation or sequestration, efflux pumping and biosorption are used by microorganisms to detoxify and immobilize metal ions such as molybdenum, chromium, copper and mercury [26–29]. Interestingly, microbial molybdate (Mo^{6+}) reduction was reported over a century ago in *E. coli* by Capaldi and Proskauer [30]. The mechanism of microbial molybdate

reduction to Mo-blue was earlier proposed by [31,32] to involve enzyme catalyzed redox changes in the oxidation state of molybdenum (M^{6+} to M^{5+}) before phosphate joins to form Mo-blue. However, the fact that similar Mo-blue spectra are formed in both bacterial molybdate reduction and ascorbate-reduce phosphate determination method, hinted on the probable formation of the phosphomolybdate intermediate [33].

To date, the enzyme responsible for molybdate reduction to Mo-blue from any bacteria although it has been purified to homogeneity, but the yield was poor. This delays the enzyme sequencing and hence the enzyme nomenclature. The term molybdenum-reducing activity was used by [31] rather than molybdenum reductase because the enzyme responsible for molybdate reduction was not previously characterized. However, it was suggested that the phenomenon is enzyme-based involving a member of oxidoreductase class. [33] purify molybdenum-reducing enzyme from *E. cloacae* Strain 48 by ammonium sulfate (40-50%) fractionation and ion-exchange on DE-cellulose and gel filtration on Sephacryl S-200. An excellent ammonium sulfate fraction with 6.5-fold enzyme purification and about 97% recovery of the initial value was achieved. SDS-PAGE of the concentrated eluates from the Sephacryl S-200 gel filtration step revealed 3 protein subunits with estimated molecular weights of 80, 90 and 100 kDa.

Recently, [33] successfully purified molybdenum-reducing enzyme from *Serratia sp*. Dr. Y5. The fraction containing enzyme activity was eluted at about 330 mM NaCl. However, by pooling, dialyzing and re-chromatographing the fraction on Mono-Q, a better separation of the enzyme fraction was achieved, obtaining a single peak 300 mM NaCl. Similarly, a single peak was obtained following the transfer of the fraction containing enzyme activity from the second ion exchange on Zorbax GF-250. Approximately, eight-fold purification was achieved following gel filtration on Sephadex G-200, contrary to EC 48 with about forty-fold partial purification. The molecular weight (apparent) of the Mo-reducing enzyme was estimated to be 105 kDa from gel filtration. A single band on Native-PAGE (after protein denaturation) at 100 kDa indicates a monomeric protein. Similarly, a marked decrease in enzyme yield results from the ion-exchange chromatography [34].

What is interesting is that based on the kinetic studies carried out on these enzymes from various sources is that the Michaelis-Menten constants varies by one order of magnitude. It is important that the constants from newer enzyme sources be characterized so that a comparison can be made, and the best enzyme be used in further improvement studies such as in genetic engineering. The study on the effect of heavy metals is also important to elucidate which heavy metal forms the strongest inhibitor to the enzyme so that effective purification strategy can be done to alleviate this inhibition. This is the aim of this study; to characterize kinetically the enzyme from a previously isolated Mo-reducing bacterium [35].

MATERIALS AND METHODS

Kinetic studies

The importance of this study is to develop an optimize enzyme reaction mixture to assay for heavy metals. Normally, the concentration of substrates must be saturating[36]. This study was carried out to determine the Michaelis constant for the Mo-reducing enzyme. The enzyme utilizes two substrates; NADH as the electron donor and phosphomolybdate as the electron acceptor. A partially purified fraction from the ion exchange fraction was used in this work. When studying the effect of electron acceptor, the reaction mixture contains 450 uL of various arbitrary concentration of LPPM of up to 5 mM (prepared as above), 60 uL of enzyme, 90 uL of NADH (final concentration of 20 mM) and 400 uL Tris.Cl pH 7.0. The final volume was 1.0 ml. The reaction was started by adding enzyme. When the K_m for LPPM was obtained (1.975 mM), the study for the determination of K_m for NADH was carried out using saturating concentration of LPPM (5 mM or approximately 5 times the K_m to get zero order reaction) [37].

Molybdenum-reducing Enzyme Assay

Molybdenum-reducing enzyme was assayed using molybdate as the electron acceptor and NADH as the electron donor [37]. Briefly, laboratory-prepared ten to four phosphomolybdate or 10:4 ratios of phosphomolybdate was prepared arbitrarily as a 60 mM stock solution in deionized water 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 30 mM (final concentration) laboratory-prepared electron acceptor substrates in 50 mM citrate-phosphate buffer pH 5.0 at room temperature, 100 µl of NADH (500 mM stock) was added to a final concentration of 50 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 the wavelength of 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 $mM.^{-1}.cm^{-1}$ [33]. 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.

Preparation of heavy metals solutions

Heavy metals and metals such as chromium (vi) ($K_2Cr_2O_7$, BDH), selenium (vi) (Na_2SeO_4, BDH), nickel (ii) ($NiCl_2$, (Ajax Chemicals), zinc (ii) ($ZnSO_4$ anhydrous J.T. Baker), iron (ii) ($(NH_4)_2Fe(SO_4)_2.6H_2O$, BDH), tungsten (vi) ($Na_2WO_4.2H_2O$, BDH), tin (ii) ($SnCl_2.2H_2O$, BDH) manganese (ii) ($MnSO_4.H_2O$, BDH), borate (iii) (H_3BO_3, anhydrous BDH), cobalt (ii) ($CoCl_2.6H_2O$, J.T. Baker), aluminium (iii) ($Al_2(SO_4)_3$, anhydrous BHD) were prepared from commercial salts or from Atomic Absorption Spectrometry standard solutions from MERCK such as mercury (ii), arsenic (v), cadmium (ii), lead (ii), copper (ii) and silver (ii).

Heavy metals were initially diluted in 0.1 M Tris.Cl buffer pH 7.0 to the final concentration of 20 mgl^{-1} to ensure that the nitric acids from the commercial heavy metals solution are neutralized. Suitable volumes of the heavy metals were then incubated with 50 µl of enzyme for 20 minutes at 4 ^oC. The mixture was then added into the enzyme reaction mixture as before. The final volume of the reaction mixture was 1 ml.

Determination of Kinetic Parameter

The effect of substrate concentration on the activity of Mo-reducing enzyme was carried out by performing standard assays at varying concentration of NADH and LPPM. Since the current trend is to use non-linear regression which is more accurate in giving K_m and V_{max} values for hyperbolic kinetic curve than the classical Lineweaver-Burke plot [38], the nonlinear regression

software GraphPad available from www.graphpad.com is utilized in this study.

Optimum pH

Molybdenum reducing enzyme activity was measured at various pH values ranging between 4.0 and 8.0 using two types of buffer. The buffers were phosphate buffer (pH 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) and Tris-HCl buffer (pH 7.0, 7.5 and 8.0) each of 50 mM. As shown in **Fig. 1**, maximum activity was obtained at pH 5.5. Shukor et al., [36] reported that, the activity of molybdenum reducing enzyme in EC 48 occurs optimally at pH 5.0. The formation of 12-MP and heteropolymolybdates in general requires an acidic environment [2,37]. This could explain the low optimum pH for the reaction of the enzyme on 12-MP for both bacteria, since the substrate is not stable at neutral and higher pH. The lower activity exhibited by phosphate buffer is possibly due to the effect of phosphate on phosphomolybdate instability.

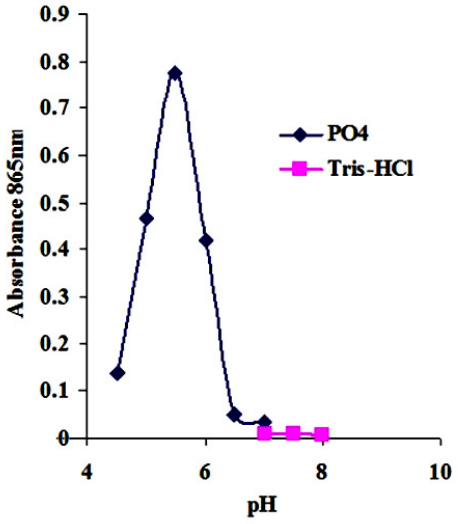

Fig. 1. Effect of pH on enzyme activity using an overlapping buffer system consisting of Tris-HCl and phosphate buffers. Error bars are mean ± standard deviation of triplicates.

Optimum temperature

The effects of temperature on Mo-reducing enzyme activity are shown in **Fig. 2**. The effects of temperature are determined at temperatures ranging from 30 to 50°C at pH 6.0 for 30 min. The Mo-reducing enzyme showed maximum activity in between 25 and 35 °C the activity drastically dropped at higher temperatures and no activity was detected higher than 50 °C. This result was comparable to the previous study of the partial purified molybdenum reducing enzyme from EC 48 with optimum activity ranging between 28 to 33 °C. The profile of optimum temperature fits well for most mesophilic bacterium with activity ranges from 20 to 40 °C.

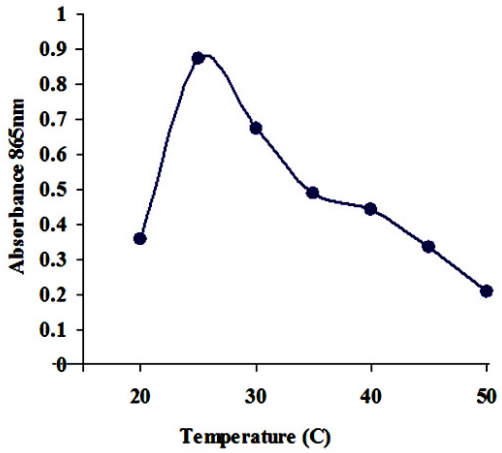

Fig. 2. Effect of temperature on enzyme activity. Error bars are mean ± standard deviation of triplicates.

Kinetic Studies Using NADH as the substrate Electron Donor

In this study, NADH serves as the substrate for electron donor and LPPM (50mM) acts as the substrate of electron acceptor [33]. The K_m and V_{max} values of these two substrates were determined using Michaelis-Menten and Lineweaver-Burk. The correlation coefficient of 0.9865 for NADH respectively suggests a good linearity. **Fig. 3** shows Michaelis-Menten plot, the K_m and V_{max} of NADH are 6.646 mM and 0.057 unit/mg enzyme. **Fig. 4** shows Lineweaver-Burk plot, the K_m and V_{max} of NADH are 7.98 mM and 0.18 unit/mg enzyme. Previous studies showed that the K_m and V_{max} of Mo-reducing enzyme from *E. cloacae* 48 was 1.65 mM and 6.28 nmole/min/mg protein. The apparent K_m for NADH was much higher compared to 1.65 mM for EC 48 suggesting lower affinity to Mo-reducing enzyme compared to EC 48.

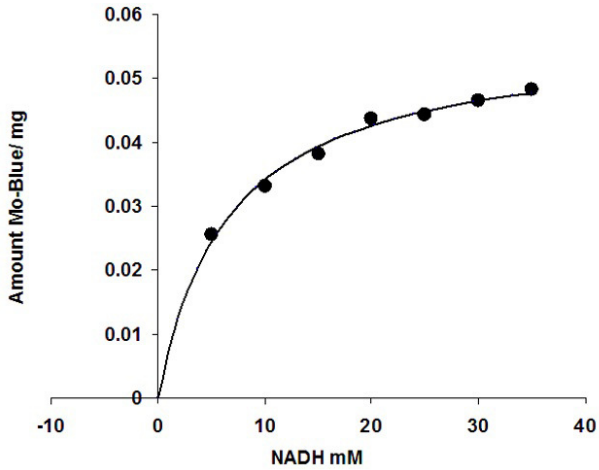

Fig. 3. Michaelis-Menten plot of initial velocity versus electron donor substrates (NADH). The concentration of the electron acceptor, LPPM, was at saturation level at all points. The error bars represent mean ± standard deviation of three replicates.

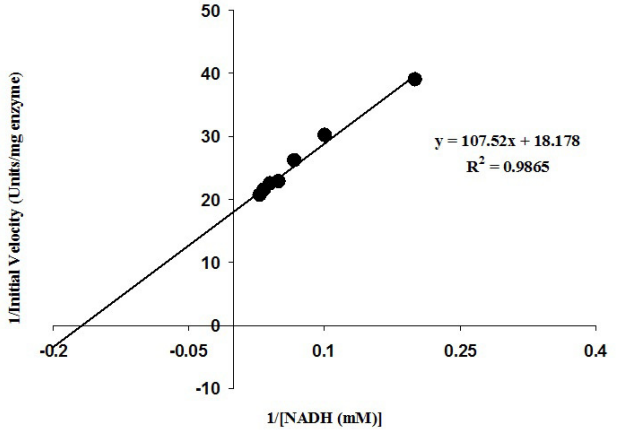

Fig. 4. Lineweaver-Burk Plot of reciprocal intial velocity at different reciprocal e donor substrate; [NADH]. The concentration of the Acceptor LPPM was at Saturation Level at all Points. The error bars represent mean ± standard deviation of three replicates.

Kinetic Studies Using LPPM as the substrate Electron Acceptor

In this study, LPPM was used as the substrate for electron acceptor and NADH (35 mM) serves as the substrate for electron donor. The Km and VmaxK_m and V_{max} values of these two substrates were determined using Michaelis-Menten and Lineweaver-Burk. The correlation coefficient of 0.9505 for LPPM respectively suggests a good linearity. **Fig. 5** shows Michaelis-Menten plot, the K_m and V_{max} of LPPM were 3.399 mM and 0.106 unit/mg enzyme. **Fig. 6** shows Lineweaver-Burk plot, the Km and Vmax of LPPM are 5.37 mM and 0.05 unit/mg enzymes.

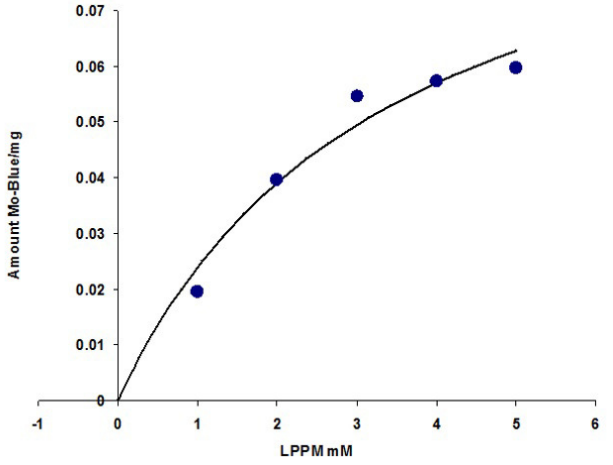

Fig. 5. Michaelis-Menten plot of initial velocity versus substrate LPPM or laboratory-prepared-10:4-phosphomolybdate. The concentration of the e- donor, NADH, was at saturation level at all points. The error bars represent mean ± standard deviation of three replicates

Previous studies showed that the K_m and V_{max} of Mo-reducing enzyme from *E. cloacae* 48 was 0.32 mM and 12.09 nmole/min/mg protein. The comparison of Mo-reducing enzyme between these two bacteria, suggested that the *E. cloacae* strain 48 has higher affinity for LPPM compared to this strain. Previously, the enzyme from the bacterium *Serratia* sp. starin MIE2 showed a K_m and V_{max} for NADH as 0.859 mM and 16.11 nmol Mo-blue/min/mg protein, respectively while the K_m and V_{max} for LPPM at 5 mM NADH was 6.02 mM and 6.89 nmol Mo-blue/min/mg protein and 6.02 mM, respectively while NADPH was not studied [39].

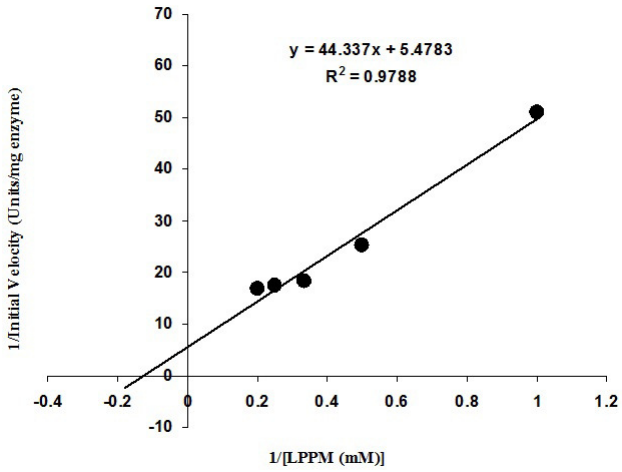

Fig. 6. Lineweaver-Burk Plot of reciprocal intial velocity at different reciprocal e acceptor substrate; [LPPM]. The concentration of the Donor NADH was at Saturation Level at all Points. The error bars represent mean ± standard deviation of three replicates.

Ahmad et al. [40] also made a similar attempt to purify molybdenum-reducing enzyme from an Antarctic bacterium (*Pseudomonas sp*. strain DRY1), about five-fold purification was achieved following gel filtration, however, the purification yield was small which is believed to be lost during gel filtration and ion exchange chromatography. Similarly, the Native-PAGE analysis reveals multiple bands indicating unsuccessful purification. The partially purified enzyme was shown to have optimum pH and temperature of 6.0 and 20 ^{o}C respectively. Although, a preliminary finding shows that 20 mM phosphomolybdate was saturating. However, at 15 mM phosphomolybdate, the plot of initial rates against concentrations of electron donor substrate revealed an apparent V_{max} for NADH as 26.98 nmol Mo-blue/min/mg protein and an apparent K_m of 4.68 mM. The Km value was smaller than that observed in this work suggesting that the Mo-reducing enzyme from this study has a lesser affinity to the enzyme from strain DRY1. In addition, the lower V_{max} value observed in this work also suggest that strain DrY1 is superior.

At 25 mM NADH (saturating NADH concentration of five times K_m), the apparent V_{max} and apparent K_m values for phosphomolybdate were 23.48 nmol/min/mg protein and 3.52 mM, respectively. However, when electron donor substrate was NADPH, the apparent V_{max} and K_m values for phosphomolybdate were 27.75 nmol/min/mg protein and 3.8 mM respectively.

Effect of Heavy Metals on Molybdenum-reducing enzyme

The results showed that the enzyme activity for *Bacillus pumilus* strain Lbna were inhibited by all heavy metals used. Zinc, copper, silver, chromium, cadmium and mercury all caused more than 50% inhibition to the Mo-reducing enzyme activity with copper being the most potent with an almost complete inhibition of enzyme activity observed (**Fig. 7**). All heavy metals are tested at the standard 1 mg/L. Shukor et al., 2009 also reported that copper can be hindered mo-reducing

enzyme from *S. marcescens* with IC_{50} values of 0.099 ± 0.013 mg/L. Inhibition mechanism of copper is still not clear, while mercury and lead may be prevented most sulfhydryl group at the active sites.

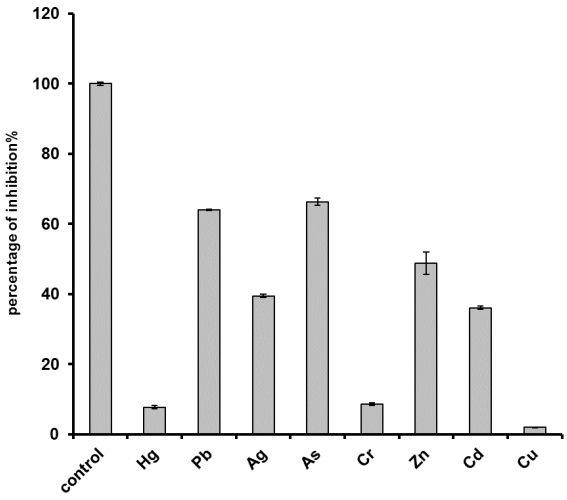

Fig. 7. Screening of eight heavy metals affecting molybdenum-reducing enzyme activity. Error bars represent mean ± standard error (n=3).

CONCLUSION

The Mo-reducing enzyme has only been purified and partially purified and characterized from a limited number of molybdenum-reducing bacteria. The characterization of the Mo-reducing enzyme from this bacterium showed limited similarity to previous Mo-reducing enzyme preparation with lower affinity and activity to other Mo-reducing enzyme and susceptibility to heavy metals which is similar to nearly all of the Mo-reducing enzyme isolated to date.

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