Microbiological reduction of hexavalent molybdenum to molybdenum blue

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In general, microbes are able to survive the toxic effects of heavy metals by using a variety of mechanisms such as bioreduction to a less toxic state, active pumping to remove heavy metals from the cytosolic environment, bioprecipitation and sequestration to name a few. Molybdenum is one of the heavy metals that have recently emerged as a significant pollutant. Molybdenum is not toxic to humans but very toxic to ruminants. Its pollution has been reported worldwide and its bioremediation has been reported. Bacterial-based remediation of molybdenum involves reduction of molybdenum into the precipitable molybdenum disulphide or to Mo-blue. The former involves bacterial production of toxic hydrogen sulphide while the latter is a nontoxic compound. Hence, molybdenum reduction to Mo-blue is a good candidate for bacterial-based bioremediation. Molybdenum reduction to Mo-blue by microbes has been reported since the last one hundred years in bacteria such as *E. coli*, *Thiobacillus ferrooxidans,* *Enterobacter cloacae*, *S. marcescens*, *Pseudomonas* sp. and *Acinetobacer calcoaceticus*. A recent work has identified phosphomolybdate as an intermediate between molybdenum and molybdenum blue, a trait seen in chromate reduction. The use of phosphomolybdate instead of molybdate accelerated the enzyme rate more than a hundred fold. The enzyme responsible for the reduction remains to be identified and its identification would be the cornerstone for this more than a one-hundred years phenomenon.

Keywords bacteria; molybdenum reduction; molybdenum blue

1. Introduction

Bacterial remediation of toxic compounds is a technology that has stirred great interest as it is generally cheaper than existing chemical and physical methods. A variety of mechanisms is used by microbes to resist the toxicity of inorganics such as heavy metals. One of these mechanisms is enzymatic reduction and it has brought about the detoxification of metal ions such as chromium, mercury, molybdenum, arsenic, lead, copper, uranium, selenium, bismuth and tungsten [1]. Among these heavy metals, molybdenum is one of the most ubiquitous metals to be found in industries. Bacterial-based remediation of molybdenum involves reduction of molybdenum into a precipitable forms, most commonly reported is to molybdenum disulphide [2] and to Mo-blue [3]. In the reduction of molybdenum to molybdenum disulphide, hydrogen sulphide, a toxic gas, is produced by the bacteria, whereas reduction to Mo-blue is mediated through enzymes. Hence molybdenum reduction to Mo-blue is a better candidate for bioremediation. Due to the toxicity of molybdenum and its emerging role in pollution, bioremediation of molybdenum has been gaining attention as a cheaper alternative to conventional chemical or physical remediation. For example, molybdenum bioremediation on a molybdenum contaminated site using a bacterial consortium in a cattle grazing land in Tyrol, Austria was successful in reducing the toxic effects of molybdenum [4]. The earliest known report on the microbial reduction of molybdenum to Mo-blue was in *E. coli* [5]. This finding, initially observed as a scientific curiosity, was later realised to be a promising tool for the bioremediation of molybdenum [3]. Since then more molybdenum-reducing (Mo-reducing) bacteria have been isolated.

2. The Chemistry and Biochemistry of Molybdenum

Molybdenum is the fourth member of the second transition series and is placed with chromium and tungsten in Group VIB of the Periodic Table. Molybdenum has an atomic number of 42 and an atomic weight of 95.94 g/mole. In its chemical properties, molybdenum resembles tungsten and vanadium, the first member of Group VB, rather than chromium [6]. Chemically, molybdenum is extremely versatile, forming compounds in a range of readily inter-convertible oxidation states. Commonly used molybdenum-oxygen compounds are molybdenum trioxide, MoO_3, sodium molybdate, $Na_2MoO_4.2H_2O$, ammonium dimolybdate, $(NH_4)_2Mo_2O_7$, and ammonium heptamolybdate, $(NH_4)_6Mo_7O_{24}\ .4H_2O$. Molybdenum (6+) or Mo^{6+} ion does not exist in solution. It exists as molybdate ions, $[MoO_4]^{2-}$ [7]. Under acidic conditions molybdate ion would combine, and form polyions such as $Mo_7O_{24}^{6-}$, $Mo_8O_{26}^{4-}$ and $Mo_{12}O_{37}^{2-}$ [8]. These polyions can be reduced by reducing agents to form "isopolymolybdenum blue". They could also combine with many heteroatoms such as; phosphate, arsenate, tungstate, sulphate, and silicate forming molybdophosphate, arsenomolybdate, tungstomolybdate, sulphomolybdate, and silicomolybdate respectively. These heteroatoms, which are situated inside "cavities" that are basket-like, consists of several tetrahedral molybdates anions joined to each other at the oxygen atom [9]. These latter compounds are known as heteropolymolybdates, which can be

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reduced by a variety of reducing agents such as dithionite, ascorbic acid and metal ions into intense blue, colloidal products known as heteropolymolybdenum blue. This phenomenon is a prominent feature of its chemistry [7].

The mechanism of heteropolymolybdate reduction to molybdenum blue or Mo-blue has been extensively studied. According to the electron spin resonance (esr) work, dithionite, a reducing agent, donates two electrons to a heteropolymolybdate, $PMo_{12}O_{40}^{3-}$ (12-molybdophosphate) converting it to Mo-blue. The introduced electrons are uniformly dispersed over the whole polymetallate sphere by a thermally activated hopping process. The electrons in the two-electron reduced forms were shown by ^{17}O nuclear magnetic resonance (nmr) spectroscopy to be very mobile, thus averaging the valence of all 12 molybdenum atoms [10]. This explains the mixed valence (between 5+ and 6+) properties of Mo-blue [6].

The scanning spectrum of the resultant Mo-blue from ascorbic acid-reduced 12-molybdophosphate show a maximum peak of between 860 and 880 nm and a characteristic shoulder approximately at 700 nm [11]. The difference between one heteropolymolybdate species to another can be seen in the scanning spectra of their reduced form (Mo-blue). Figure 2 shows the scanning spectra of various reduced heteropolymolybdates whilst Figure 3 shows the structure of a 12-molybdophosphate.

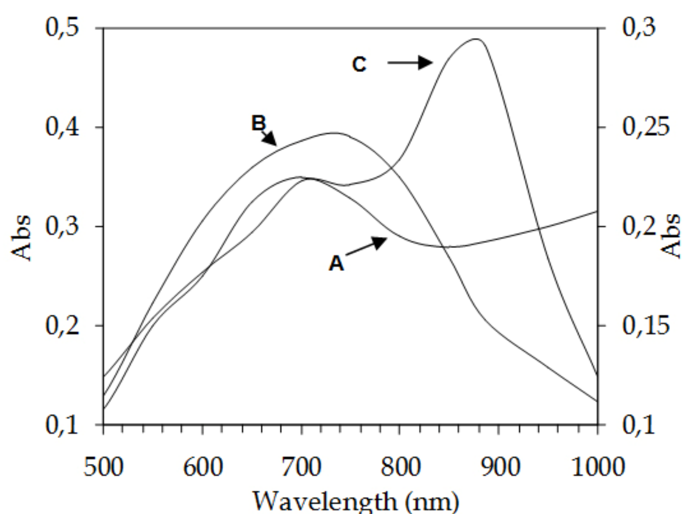

Fig.1. Scanning spectra of Mo-blue from molybdosilicate (A), molybdosulphate (B) and molybdophosphate (C).

Molybdenum is essential to plant growth as a component of the nitrogen fixation enzymes; nitrate reductase, nitrite reductase and nitrogenase, which are found in plant organs called legumes. The mechanism of nitrogen fixation in enzymes and in model systems *in vitro* has been extensively investigated [12]. The microorganisms that fix molecular nitrogen fall into two classes. The first is the symbiotic microorganisms that fix nitrogen in association with plants, e.g., *Rhizobium* and the second is the asymbiotic microorganisms that are free-living and include *Azotobacter vinelandii* and *Clostridium pasteurianum.* From cell-free extracts of *C. pasteurianum*, two metalloproteins have been obtained. One of the two is the hydrogen donating system, azoferredoxin, which contains iron and sulphide and the other is the nitrogen activating system, molybdoferredoxin, which contains molybdenum, iron, and sulphide [13]. Molybdenum is an essential constituent of the enzymes xanthine oxidase (XOD) and aldehyde oxidase (AOD), which occur in the liver and intestines, and hepatic sulphite oxidase. XOD has been isolated from cow's milk. The enzyme oxidizes hypoxanthine to xanthine and xanthine to uric acid in purine catabolism. XOD has a broad spectrum of substrates and will catalyse the oxidation of many purines and aldehydes with very different rates of reaction [14].

3. Molybdenum toxicity

Molybdenum toxicity, like all compounds, is assessed according to an acute or chronic aspect. It is also important to assess the toxicity of molybdenum compounds on several different species since many chemicals are species specific in their action of toxicity. Thus, toxicity studies of molybdenum compounds such as molybdenum trioxide, ammonium and calcium molybdates and molybdenum disulphide have been carried out with rats and guinea pigs by the U.S. Public Health Service [15]. Molybdenum trioxide, when fed in large daily doses of from 1200 to 6000 mg Mo/kg body weight has been proven to be fatal. Fatalities were few at doses of 120 to 600 mg. Molybdenum disulphide did not produce fatal results. The highest dose in rats and guinea pigs, when extrapolated, corresponds to 420 g for a 70 kg human being.

In inhalation experiments (5 mg Mo/cubic feet of air), molybdenum trioxide and ammonium dimolybdate were injurious but molybdenum disulphide was much less so. Most molybdates compounds also produced toxic effects when administered orally and by intraperitoneal injection in large doses (400 to 800 mg/kg). Experiments on farm animals supplemented with sodium molybdate ranging from 20 to 1000 mg Mo/kg body weight shows that cows are the least tolerant with drastic scouring at 20-50 mg Mo/kg body weight followed by sheep and pigs. Horses are the most tolerant, with no significant health effect after molybdenum supplementation at 1000 mg Mo/kg body weight for three months. Ill effects in cows range from hypocupraemia, lameness, osteoporosis and spontaneous bone fractures. It was later found that an excess amount of molybdenum in the diet causes an antagonistic decrease of copper, another important enzyme cofactor and the addition of copper ions can result in a complete recovery from the signs of molybdenum intoxication [16].

4. Molybdenum usage and pollution

Molybdenum's diversity has proven precious in industries. Its uses include super alloys, nickel base alloys, lubricants, chemicals, glass workings, ink, pigments, electronics and many other applications. It is from these industries that molybdenum can be found in the discharged effluents [17]. In the Tokyo Bay and the Black Sea, molybdenum level is in the range of hundreds of ppm (parts per million) making it a significant pollutant [18]. Molybdenum pollution can come from various other sources including geologic. For example, the stream sediments of the Ten Mile Creek in Colorado contain between 20 to 2000 ppm total molybdenum [19]. Pollution due to molybdenum has not often been reported due to its low toxicity to humans despite its significant toxicity towards ruminants at several parts per million [16].

5. Major problem in microbial reduction of molybdenum to molybdenum blue

Bacteria and many reducing agents, biotic and non-abiotic can reduce molybdenum to Mo-blue. This has caused problems in proving a biological and physiological basis of molybdate reduction in microbes [20,21]. This problem is not confined to molybdenum. Such is the problem with nonenzymatic reduction of metals that early studies of ferric reduction have been suggested to be non-enzymatic. An ingenious way was developed by using dialysis tube to prove that the reduction of the metal iron (Fe^{3+} to Fe^{2+}) by the bacterium *Clostridium butyricum* was catalyzed enzymatically. Munch and Ottow demonstrated that the reduction of Fe^{3+} in the form of Fe_2O_3 (hematite) to Fe^{2+} was indeed caused directly by enzymatic reaction and not due to Fe-reducing chemicals [22]. Borrowing this idea, we found out that all of the recently isolated heterotrophic Mo-reducing bacterium uses enzymatic action to reduce molybdenum [23-29,30].

6. Microbes that reduces molybdenum to molybdenum blue

Molybdenum reduction to Mo-blue by microbes has been reported since the last one hundred years [1,5,31-33]. Detailed studies of Mo-reduction such as the effect of molybdate and phosphate concentrations, heavy metals and absorption spectrum are initiated in *E. coli* K12 by [34]. In 1988, Sugio *et al.* reported on the reduction of molybdate into Mo-blue by *Thiobacillus ferrooxidans* strain AP19-3 (now *Acidithiobacillus ferrooxidans* strain AP19-3)[35]. However, further studies by Yong *et al.* [36] showed that aside from enzymatic reduction, Fe^{2+}, which is present in the media, is a dominant factor in reducing the molybdenum to Mo-blue. The scarcity of the report on microbial molybdate reduction is demonstrated in the citational absence of Campbell *et al.* [34] works by Sugio *et al.* [35], Ghani *et al.* [3], Ariff *et al.* [37] and Lloyd [38]. Recently, more Mo-reducing bacteria were reported. These include *Serratia marcescens* strain Dr.Y6 [24], *Serratia* sp. strain DRY5 [25], *Enterobacter* sp. strain DRY13 [26], *Serratia* sp. strain Dr.Y8 [27], *S. marcescens* strain DRY9 [28], *Acinetobacter calcoaceticus* [29] and *Pseudomonas* sp. strain DRY2 [30]. We also have isolated several gram positive Mo-reducing bacteria such as *Staphylococcus* sp. and *Bacillus* sp. (Unpublished works). We observed that in all of these Mo-reducing bacteria, a general requirement of either glucose or sucrose as a electron donor source, pH of between 6.0 and 8.0, optimal temperatures between 30 and 37 ^{o}C, and molybdenum concentrations between 20 and 50 mM. Generally, phosphate concentrations above 5 mM are inhibitory. High phosphate inhibits molybdate reduction by maintaining the pH at neutral; a pH that is undesirable for the formation and stability of phosphomolybdate [7,39]. All of the heterotrophic Mo-reducing bacteria isolated so far requires static conditions for maximum production of Mo-blue.

7. Mechanism of molybdenum reduction to molybdenum blue

The mechanism of molybdate reduction to Mo-blue by the heterotrophic microbes has been suggested to involve the enzymatic reduction of molybdate (Mo^{6+}) to molybdenum (v) ions before addition of phosphate ions leads to the formation of the Mo-blue [3] as shown in Figure 2. However, we proposed that there is an intermediate between molybdate and Mo-blue based on the observation of the spectrum of the Mo-blue produced by all of the heterotophic Mo-reducing bacteria

isolated so far [24-30,40] with a peak maximum at 865 nm and a shoulder at 700 nm (Figure 3). This spectrum is very similar to the Mo-blue produced from the phosphate determination method [11]. It has been known that Mo-blue produced from the phosphate determination method is reduced phosphomolybdate [10,41], hence, we suggested that phosphomolybdate is an intermediate species in molybdenum reduction in all of the heretotrophic bacteria [40]. Moreover there are enzymes that are capable of reducing phosphomolybdate but not molybdate to Mo-blue [39]. Campbell *et al.* [34] were the first to suggest that the possible identity of Mo-blue from the reduction of molybdate by *E. coli* K12 is a reduced phosphomolybdate. Although identification of phosphomolybdate by analyzing the scanning spectroscopic profile would not be enough to distinguish the many subtypes and lacunary species of phosphomolybdate but is generally an accepted method to distinguish between one heteropolymolybdate to another e.g. between phosphomolybdate and silicomolybdate or sulfomolybdate [10,41]. Hence, a more plausible mechanism is shown in Figure 4. The requirement for an intermediate species in molybdate reduction is also shared by chromate, a chemically related ion [7,42,43].

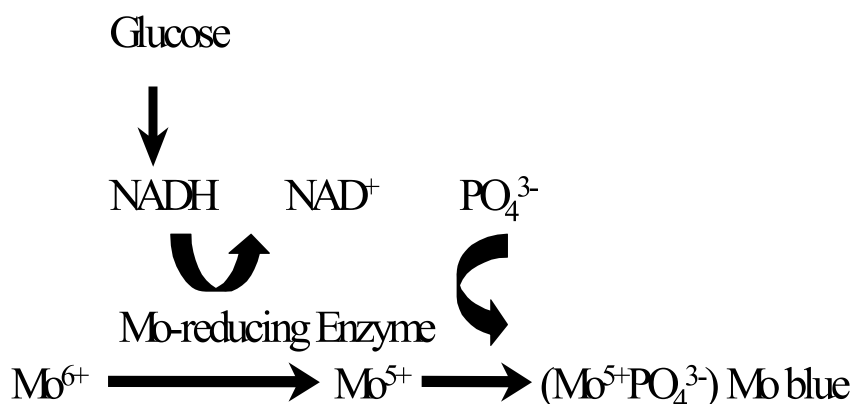

Fig. 2. A schematic presentation of the mechanism of molybdate reduction to Mo-blue by EC 48 (modified from Ghani *et al.* [3]).

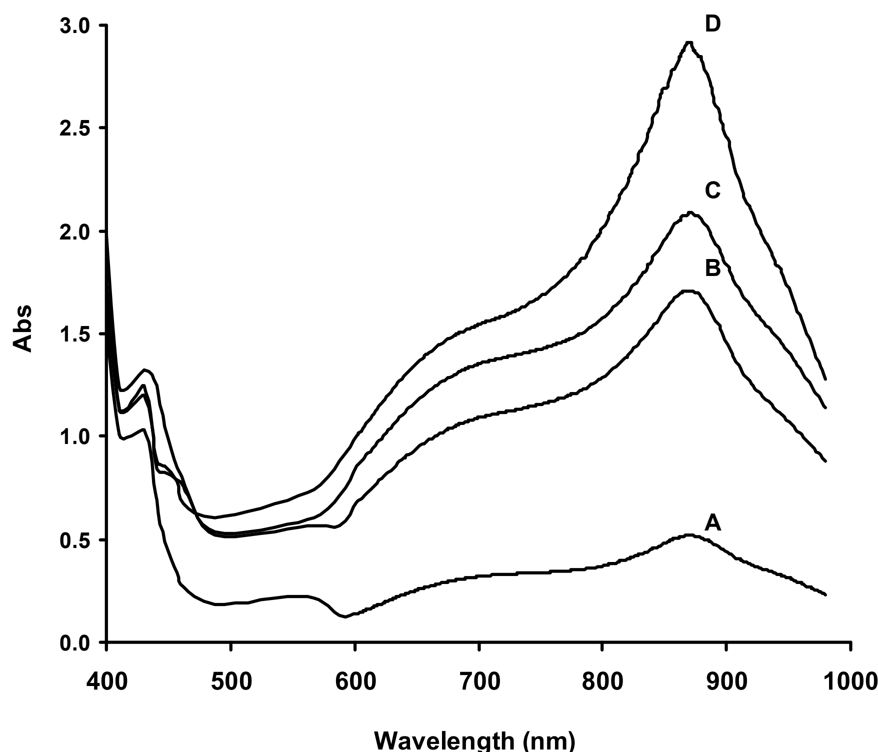

Fig. 3. Scanning spectra of Mo-blue from *Pseudomonas sp.* strain DRY2 after 14, 16, 18 and 20 hr of static incubation labelled A, B, C and D, respectively (Unpublished works).

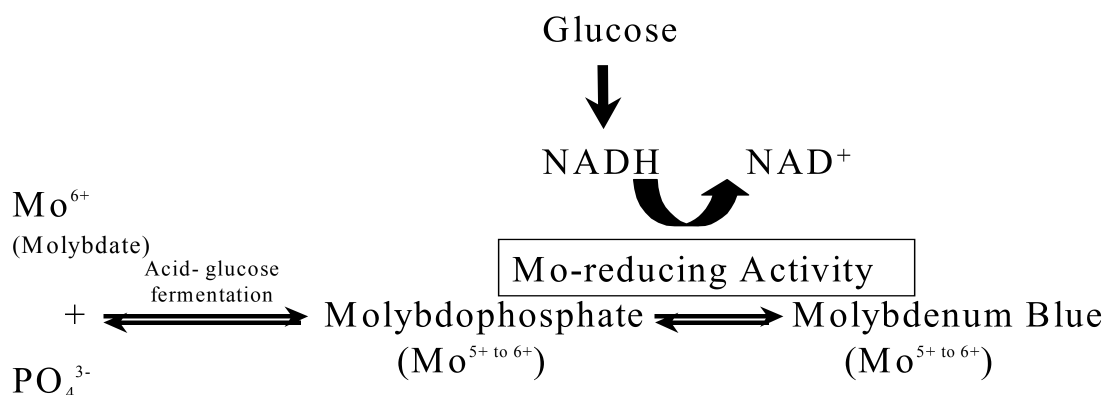

Fig. 4. A new suggested mechanism for molybdate reduction to Mo-blue in bacteria (from [40]).

The discovery of phosphomolybdate as an important intermediate has also improved the Mo-reducing enzyme assay. Previously, molybdate is used as the electron acceptor in the assay and the activity was low [3]. An attempt to purify this enzyme beyond ammonium sulphate fractionation proved unsuccessful [37]. Since phosphomolybdate is shown to be an important intermediate species, we proceed to develop a new enzyme assay based on 12-phosphomolybdate (12-phoshomolybdate) as the electron acceptor and found the activity increased several hundred folds. In addition, the assay can be carried out in an open-cuvette manner while the original assay requires complete anaerobicity. Using this assay allowed us to partially purify the molybdenum reducing activity using ion exchange and gel filtration chromatography [44]. More recently, we improve the enzyme assay using laboratory-prepared phosphomolybdate instead of the commercial 12-molybdophosphate [45]. Using this new substrate we successfully purified the Mo-reducing enzyme from *S. marcescens* strain Dr.Y5. The enzyme was purified after anion exchange and gel filtration chromatography on Mono Q and Zorbax GF-250 columns, respectively. The monomeric enzyme has an apparent molecular weight of 105 kDalton (Unpublished works).

8. Effects of respiratory inhibitors and heavy metals on molybdenum reduction

The use of cyanide as a respiratory inhibitor and TMPD as an artificial electron donor in EC 48 suggest that the site for molybdate reduction is downstream from cytochrome b [3]. In contrast, respiratory inhibitors such as antimycin A, rotenone, sodium azide and cyanide tested to several of the recently isolated Mo-reducing bacteria did not show any inhibition to the Mo-reducing activity [24-30]. The results suggest that the electron transport system maybe is not the site of molybdenum reduction to molybdenum blue in bacteria. The use of respiratory inhibitors to probe location of metal-reducing or oxidizing enzyme is known to show mixed results. Respiratory inhibitors such as rotenone, azide and cyanide failed to inhibit chromate reduction in *E. coli* [46] and in *Pseudomonas mendocina* [47] while cyanide and azide inhibit the reduction of chromate in *Bacillus subtilis* [48].

Some metals such as copper, chromium, selenium, zinc, nickel and cobalt are essential at certain concentrations and become toxic at high concentrations while other metals such as mercury, lead, cadmium, silver and arsenate are toxic even at low concentrations. In bioremediation, resistance to all of the above metals is desired and microbes are able to do this by a variety of mechanisms. Among the metal ions tested, ferrous and stannous ions were found to significantly increase the activity of Mo-reducing activity in all of the Mo-reducing bacterium [24-30]. However, ferrous and stannous ions were later found to chemically reduce molybdate to Mo-blue without the participation of enzyme. It is well known that heteropolymolybdate ions are easily reduced to Mo-blue by reducing agents such as stannous, dithionite and ferrous ions [6]. Even the stannous ions were used as a chemical reductant for the construction of the Mo-blue standard curve [3,35]. This is likely the reason for the reduction of molybdate to Mo-blue in the acidic media of *T. ferrooxidans* [36] and in all of the other Mo-reducing bacteria studied. Other metal ions such as chromium, cadmium, copper, mercury and lead caused significant inhibition in all of the Mo-reducing bacterium studied. The problems of chemical contribution on molybdenum reduction have lead us to develop a method to study this effect using ascorbic acid as a reducing agent of phosphomolybdate and found that the inhibitory effects of phosphate and arsenate to Mo-blue production is not at the cellular level but their mode of inhibition is possibly by disrupting the heteropolymolybdate complex. Only mercury was found to be a physiological inhibitor towards molybdate reduction in EC 48 [23].

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9 Conclusions

Molybdenum-reduction to molybdenum blue is a phenomenon that has baffled scientists for more than one hundred years. Only recently that it is proven to be a physiological activity catalysed by enzyme and not by biotic chemical reductants. Its usage as a potential bioremediation tool in molybdenum-contaminated areas has resulted in the isolation of many efficient Mo-reducing bacteria. Knowledge of the various optimized parameters for these bacteria would facilitate an easy and more effective translation of the laboratory results to the fields. The mechanism of its reduction has been revealed to involve a preliminary formation of an intermediate species, phosphomolybdate, via chemical route followed by the enzymatic reduction to molybdenum blue. Important future works would be to localise the site of reduction since a cytosolic location would imply transport problem due to the large molecular structure of the reduced phosphomolybdate. In addition, it is important to screen for more tolerant strains as sites with metal contamination usually contain an assortment of toxic heavy. Finally, the purification of the Mo-reducing enzyme followed by its sequencing is important in giving an official name to the enzyme. This would be an important milestone to the study of molybdenum-reduction in bacteria.

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