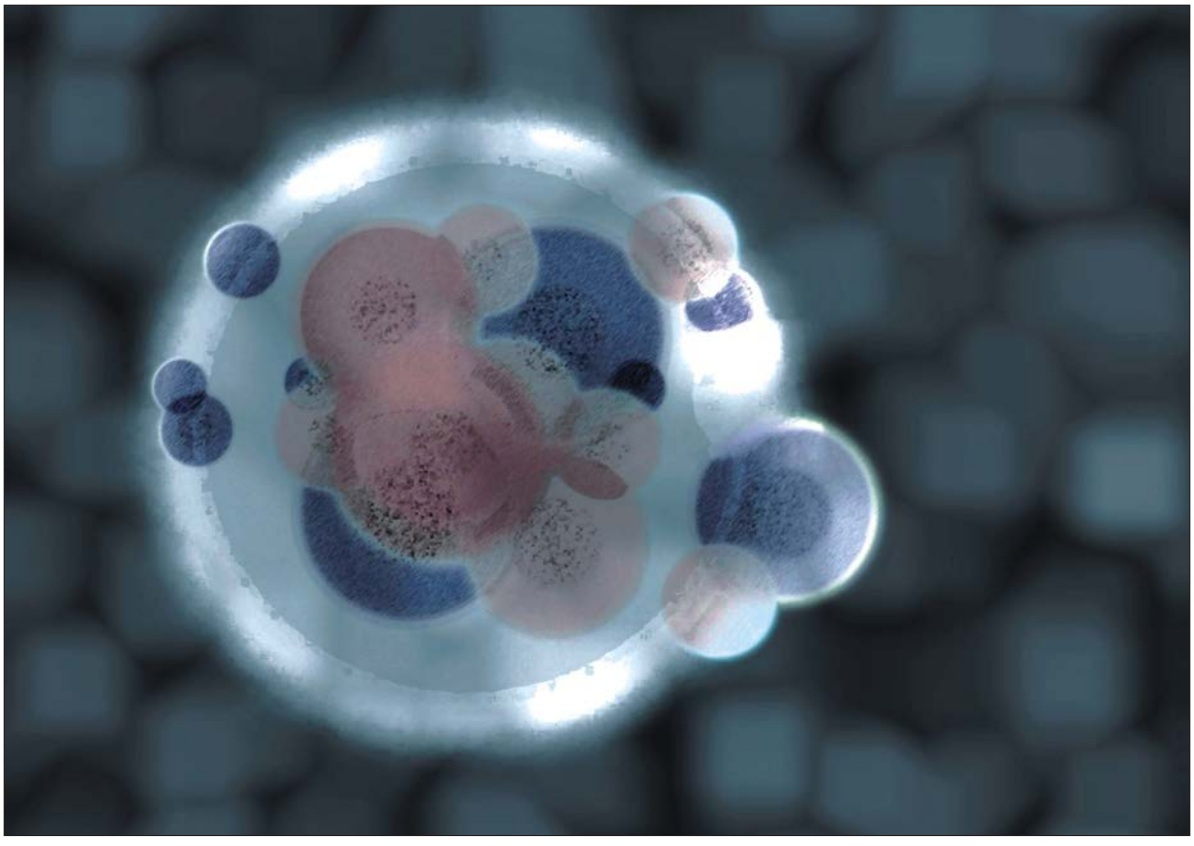

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# Molybdate Reduction to Molybdenum Blue in Microbe Proceeds via a Phosphomolybdate Intermediate

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**Abstract:** The involvement of phosphomolybdate as the intermediate during microbial reduction of molybdate to molybdenum blue is a central theme of this study. A profile of pH and molybdenum blue changes during growth of bacteria at low phosphate show that the pH drops steadily to between 5 and 6 during the initial course of bacterial growth; an event important for the conversion of molybdate to a heteropolymolybdate form, before molybdate reduction to molybdenum blue starts to increase after approximately 40 h of static incubation. During this period it was observed that the colour of the media progresses from yellow to green and finally to blue. The molybdenum blue spectra from 600-950 nm from all of the bacteria studied show similar peak maxima in between 860 and 870 nm and a shoulder at 700 nm. The overall scanning profile was found to be very similar to the scanning profile of molybdenum blue from ascorbic acid-reduced phosphomolybdate. The results obtained suggest that the identity of molybdenum blue from heterotrophic bacteria is a reduced form of phosphomolybdate.

**Key words:** Bacteria, molybdate reduction, molybdenum blue, phosphomolybdate

## INTRODUCTION

Molybdate reduction to molybdenum blue by microbes is an old phenomenon. According to Levine (1925), the phenomenon was first reported in *E. coli* by Capaldi and Proskaue (1896). In 1985, microbial molybdate reduction resurfaced again in a report on its reduction by *E. coli* K12 (Campbell *et al*., 1985). Sugio *et al*. (1987, 1988) reported the reduction of molybdate into molybdenum blue by *Thiobacillus ferreoxidans*. The efforts of Sugio *et al*. (1988) were followed by Ghani *et al*. (1993), who reported that another heterotrophic bacterium, *Enterobacter cloacae* strain 48 (EC 48) was able to reduce molybdate to molybdenum blue. Literature search showed that the study of molybdate reduction to molybdenum blue is limited and scarce and the latest study is in EC 48. A recent review on metal reduction by microbes has also confirmed this fact (Lloyd, 2003). It was initially proposed that molybdate ($Mo^{6+}$) was first reduced to $Mo^{5+}$ by molybdenum reductase (Mo-reducing enzyme) prior to the joining of phosphate anions forming molybdenum blue (Sugio *et al*., 1988; Ghani *et al*., 1993; Ariff *et al*., 1997). However this mechanism of molybdate reduction is not plausible taking into account of molybdate chemistry. A new mechanism of molybdate reduction in EC 48 was proposed involving phosphomolybdate as an intermediate between molybdate and molybdenum blue. This mechanism was proposed because the spectrum of molybdenum blue from EC 48 closely matches to the molybdenum blue from the phosphate determination method which is known to be a reduced phosphomolybdate (Shukor *et al*., 2000). In addition, it was found that there is a long lag period where pH drops to pH 5.5 before molybdate reduction occurs. This lowering of pH and in the presence of phosphate ions would transform the molybdate to phosphomolybdate (Glenn and Crane, 1956; Lee, 1977). We hypothesize that molybdate reduction in microbes especially in heterotrophic bacteria proceeds via a phosphomolybdate intermediate and not just an exclusive event seen in EC 48.

In order to corroborate this hypothesis, the spectrum of the resultant molybdenum blue from other molybdenum-reducing bacterium must match to the spectrum of the molybdenum blue from the phosphate determination method and to EC 48. In addition, the pH changes during reduction should be monitored in order to correlate the pH profile with molybdenum blue synthesis.

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A lowering of pH should be observed prior to reduction of molybdate to molybdenum blue to support the phosphomolybdate intermediate hypothesis. In this study we provide such evidences from the works involving several locally isolated heterotrophic molybdenum-reducing bacteria from different genus.

## MATERIALS AND METHODS

**Isolation and identification of molybdenum-reducing bacteria:** *Pseudomonas* sp. Strain Dr.Y2, *Serratia marcescens* Strain Dr.Y4, *Staphylococcus aureus* Strain Dr.Y11 and *Acinetobacter* sp. Strain Dr.Y12 were isolated locally throughout Malaysia and were shown to be capable of reducing molybdate to molybdenum blue. *Enterobacter cloacae* Strain 48 was originally isolated from Chengkau, Malaysia (Ghani *et al*., 1993).

Identification at species level was performed by using Biolog GN MicroPlate (Biolog, Hayward, CA, USA) according to the manufacturer's instructions and 16s rRNA molecular phylogenetics studies. Isolation of genomic, PCR conditions for the bacteria, Biolog and molecular phylogenetics studies will be presented elsewhere. The GenBank accession numbers of the partial 16s rRNA genes for the molybdate-reducing *Pseudomonas* sp. Strain Dr.Y2, *Serratia marcescens* Strain Dr.Y4, *Staphylococcus aureus* Strain Dr.Y11 and *Acinetobacter* sp. Strain Dr.Y12 are DQ226203, DQ226205, DQ226212 and DQ226213, respectively.

All bacteria were grown on agar plate and in low phosphate (2.9 mM phosphate) liquid media (pH 7.0) containing glucose (1%), $(NH_4)_2SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.5%), yeast extract (0.05%), $Na_2MoO_4.2H_2O$ (0.242%) and $Na_2HPO_4$ (0.05%). Samples were removed aseptically and the pH determined. Molybdenum blue production was monitored at 865 nm (Shukor *et al*., 2000). Suitable samples showing presence of molybdenum blue from each bacterial culture media were scanned from 500 to 950 nm (Cintra 5) with distilled water to correct the baseline.

**Preparation of ascorbic acid-reduced phosphomolybdate:** 12-phosphorous molybdate (12-MP) or phosphomolybdate (Sigma) was prepared in distilled water as a 5 mM stock solution and the pH adjusted to pH 5.0 with HCl. Ascorbic acid (Sigma) was prepared as a 25% solution in distilled water and kept at 4°C for a maximum of one week. One hundred microlitres from the 12-MP stock solutions was added to 100 µL ascorbic acid and the final volume adjusted to one milliliter with distilled water. After 12 h of incubation, the resulting molybdenum blue was scanned from 500-950 nm (Cintra 5) with distilled water to correct the baseline.

## RESULTS AND DISCUSSION

In all of the cases it can be seen that the pH drops steadily to pH between 5 and 6 during the initial course of bacterial growth before a dramatic increase in molybdenum blue production occurs (Fig. 1). Molybdate reduction to molybdenum blue starts to increase after approximately 40 h of static incubation. During this period it was observed that the colour of the media progresses from a yellowish appearance to green and finally to blue. The pH profile data suggest that acidification of the media is an important event prior to molybdenum blue synthesis in bacteria. Similar results were observed in EC 48 (Shukor *et al*., 2000). If high phosphate (100 mM) at pH 7.5 were used, the strong buffering capacity would prevent acidification and is possibly the cause of the inhibition of molybdenum blue formation in EC 48 and *E. coli* K12. This phenomenon was also observed by Glenn and Crane (1956). When they adjusted the assay media to neutral (pH 7.0) using 100 mM phosphate, phosphomolybdate reduction to Mo blue was inhibited. It is known that lowering in pH is important for molybdate conversion to phosphomolybdate prior to the latter's reduction to molybdenum blue (Hori *et al*., 1988; Lee, 1977). These evidences supports our hypothesis that molybdate reduction in microbe must proceed via a phosphomolybdate intermediate before reduction to molybdenum blue can take place.

The spectra in Fig. 2 shows the scanning profile of molybdenum blue produced by bacterial fermentation scanned from the 600 nm to the far-red region of 950 nm. There is a peak between 860 and 870 nm and a shoulder at 700 nm. The absorbance at 710 nm is approximately 30% less than at 865 nm. The overall scanning profile is very similar to the scanning profile of molybdenum blue from ascorbic acid-reduced phosphomolybdate. The latter has been suggested as a reduced form of the 12-phosphomolybdate species (Glenn and Crane, 1956; Sims, 1961; Kazansky and Fedotov, 1980; Campbell *et al*., 1985; Yoshimura *et al*., 1986; Tosi *et al*., 1998). The similarity suggests that the identity of molybdenum blue from bacteria is a reduced form of phosphomolybdate. Although identification of the exact phosphomolybdate species must be carried out using NMR and ESR (Shukor *et al*., 2000), characterization of heteropolymolybdate species by analysing the scanning spectroscopic profile is one of the less cumbersome and accepted method (Glenn and Crane, 1956; Sims, 1961; Kazansky and Fedotov, 1980; Yoshimura *et al*., 1986; Hori *et al*., 1988). Although the information would not be enough to distinguish the many subtypes and lacunary species of phosphomolybdate, it would generally be enough to distinguish between one heteropolymolybdate

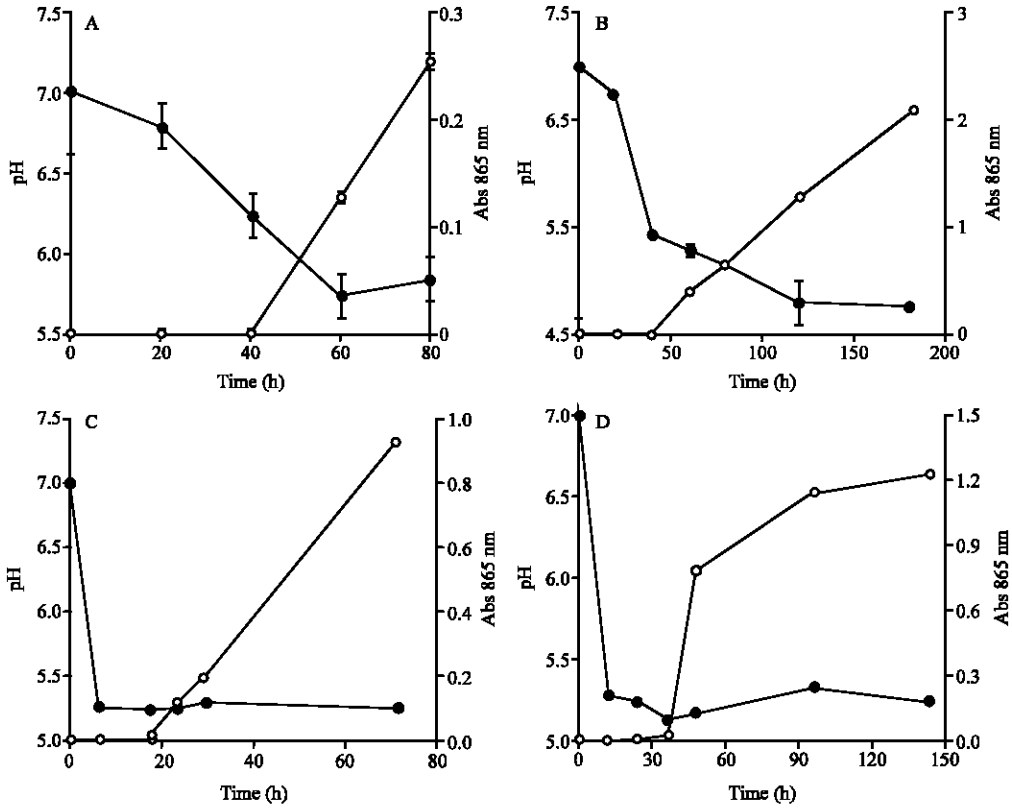


Fig. 1: Profile of production of molybdenum blue (○) and changes in pH (●) during growth under low phosphate media containing 10 mM molybdate for Bacterium Strain Dr.Y13 (A), *Klebsiella oxytoca* Strain Dr.Y14 (B), *Acinetobacter* sp. Strain Dr.Y12 (C) and *Serratia marcescens* Strain Dr.Y10 (D). The error bars represent mean±standard deviation for three replicates

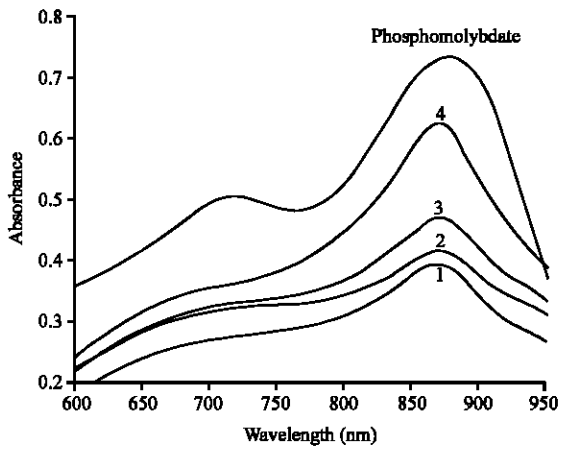


Fig. 2: Scanning spectra of molybdenum blue from *Serratia marcescens* Strain Dr.Y10 (1), *Acinetobacter* sp. Strain Dr.Y12 (2), *Klebsiella oxytoca* Strain Dr.Y14 (3) and Bacterium Strain Dr.Y13 (4) in comparison with ascorbic acid-reduced phosphomolybdate

to another e.g., between phosphomolybdate, silicomolybdate and molybdosulphate (Shukor *et al*., 2000). The difference between one heteropolymolybdate species to another can be seen in the scanning spectra of their reduced form (Fig. 3).

The implication of this finding is important in several ways. Firstly, the previous mechanism of molybdate (phosphomolybdate) reduction to molybdenum blue as suggested by Sugio *et al*. (1988) and Ghani *et al*. (1993) needs revision. They suggest that the molybdate (molybdenum $6^+$) is first enzymatically reduced to molybdenum $5^+$ before addition of phosphate leads to the formation of the molybdenum blue. We suggest that molybdate under fermenting acidic environment and in the presence of phosphate ions is converted to phosphomolybdate first before enzymatic reduction of the phosphomolybdate by microbes takes place (Fig. 4). This suggests that the enzymatic reduction mechanism would closely resemble phosphomolybdate reduction by xanthine oxidase and aldehyde oxidase (Glenn and Crane, 1956). Secondly, molybdenum blue from bacteria should

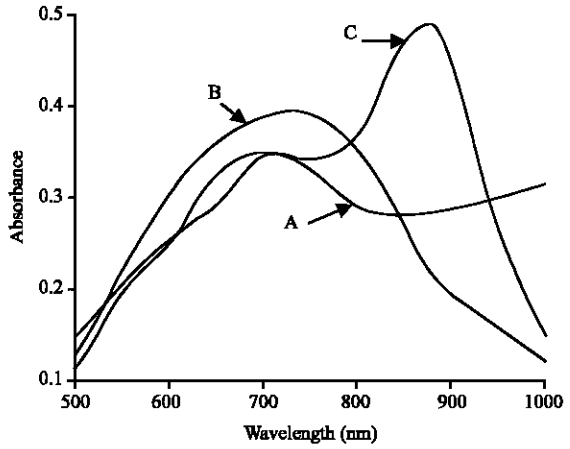


Fig. 3: Scanning spectra of molybdenum blue from molybdosilicate (A) adapted and modified from Glenn and Crane (1956), molybdosulphate (B) adapted and modified from Hori *et al.* (1988) and phosphomolybdate (C) adapted and modified from Yoshimura *et al.* (1986)

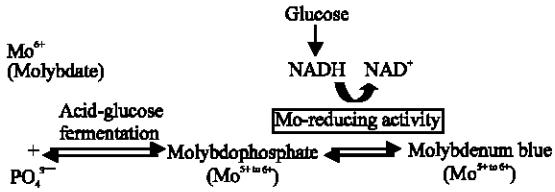


Fig. 4: A new suggested mechanism for molybdate reduction to molybdenum blue in bacteria

be quantified using phosphomolybdate as a reference and not molybdate as was used earlier by Ghani *et al.* (1993). In quantifying molybdenum blue produced in EC 48, we reduced an increasing amount of phosphomolybdate with ascorbic acid and found the increase in blue intensity linearly correlate with phosphomolybdate concentrations in the reaction mixture. We suggest the molybdenum blue assay (Shukor *et al.*, 2000) to be used as a standard method to quantify molybdenum blue from bacteria. Finally, the assay for Mo-reducing enzyme should also employ phosphomolybdate as an electron acceptor substrate as was shown in the XOD and AOD assays (Glenn and Crane, 1956) instead of molybdate as in the previous assay (Ghani *et al.*, 1993). The Mo-reducing enzyme should be assayed at the wavelength showing the maximum peak, which is at 865 nm instead of 710 nm as was employed by Ghani *et al.* (1993). Using the assay we manage to partially purify the molybdenum reducing enzyme from EC 48 (Shukor *et al.*, 2003).

Molybdenum (6+) or $Mo^{6+}$ ion does not exist in solution. It exists as molybdate ions, [$MoO_4^{2-}$] (Lee, 1977). Under acidic conditions molybdate ion would combine and form polyions such as $Mo_7O_{24}^{6-}$, $Mo_8O_{26}^{4-}$ and $Mo_{12}O_{37}^{2-}$ (Braithwaite, 1981). 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 phosphomolybdate, 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 (Greenwood and Earnshaw, 1984). These latter compounds are known as heteropolymolybdates, which can be 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 (Lee, 1977).

The mechanism of heteropolymolybdate reduction to molybdenum blue or molybdenum 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-phosphomolybdate) converting it to molybdenum 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 (Kazansky and Fedotov, 1980). This explains the mixed valence (between $5^+$ and $6^+$) properties of molybdenum blue (Sidgwick, 1984).

## CONCLUSIONS

We propose a new hypothesis on the mechanism of heterotrophic microbial reduction of molybdate to molybdenum blue. We suggest that phosphomolybdate is the intermediate that must form before reduction can take place. We showed some evidences for the new hypothesis. We have suggested in earlier publications that the enzyme assay for molybdenum reducing activity in EC 48 must use phosphomolybdate as an electron acceptor substrate and the use have resulted in a partial purification and characterization of the enzyme. Based on the findings in this study we suggest that the molybdenum reducing enzyme assay in the heterotrophic microorganism should use phosphomolybdate as a substrate. We are currently using the improved assay in purifying molybdenum reducing enzymes from the bacteria studied in this study.

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