**Pantoea sp. strain HMY-P4 Reduced Toxic Hexavalent Molybdenum to Insoluble Molybdenum Blue**

Idris, D.\(^1\), Gafasa, M.A.\(^1\), Ibrahim, S.S.\(^1\), Babandi, A.\(^1\), Shehu, D.\(^1\), Ya’u, M.\(^1\), Babagana, K.\(^1\), Mashi, J.A.\(^1\) and Yakasai, H.M.\(^1\)*

\(^1\)Department of Biochemistry, Faculty of Basic Medical Sciences, College of Health Science, Bayero University Kano, Nigeria.

*Corresponding author:
Dr. Hafeez Muhammad Yakasai,
Department of Biochemistry,
Faculty of Basic Medical Sciences,
College of Health Science,
Bayero University, Kano P. M. B 3011, Kano State,
Nigeria.
Tel: +2348034966925
Email: hmyakasai.bch@buk.edu.ng

**INTRODUCTION**

Pollution of the environment keeps on increasing at an alarming rate due to the activities of man such as urbanization, technological advancement, unsafe agricultural practices and rapid industrialization which degrades the environment. Heavy metals released into the environment are persistent due to their toxicity which poses a severe threat to organisms exposed to high levels of such pollutants. Metals are essential to the biological functions of plants and animals but at elevated levels, they interfere with metabolic reactions in systems of organisms. Toxic heavy metals e.g molybdenum which is useful to plants, are capable of reducing plant growth due to reduced photosynthetic activities, plant mineral nutrition, and reduced activity of essential enzymes [1].

Toxic heavy metals like molybdenum could accumulate in the body when consumed in contaminated food through the food chain and become health risks to living organisms [2]. This causes oxidative stress, an unevenness involving the production of free radicals and the capacity of cells to eradicate them or repair the damage [3, 4]. This leads to base damage through formation of reactive oxygen species (ROS) which includes oxygen radicals like superoxide and hydroxyl and non-radical derivatives of molecular oxygen (O\(_2\)) such as hydrogen peroxide (H\(_2\)O\(_2\)), as well as breakage of the DNA molecule [3, 4]. Furthermore, heavy metal toxicity increases the production of ROS thereby decreasing the antioxidant systems (glutathione, superoxide dismutase, etc.) which protect cells. If this condition continues, the normal functioning of the organism is affected and may invariably lead to cell death.

Molybdate reduction to molybdenum blue is an old phenomenon that was reported over a century ago. According to Levin [5], the phenomenon was first reported in *E. coli* by capaldi and proskaue [6]. In 1985, microbial molybate reduction resurfaced again in a report on its reduction by *E. coli* K12 [7]. Sugio et al. [8] reported the reduction of molybdate to molybdenum blue by *Thiobacillus ferrooxidans*. The effort of Sugio et al. [8] were followed by Ghani et al. [9], who reported on another heterotrophic bacterium *Enterobacter cloacae* strain EC48 (EC48) was able to reduce molybdate to molybdenum blue.
A review on metal reduction by microbes has also confirmed this fact [10]. It was initially proposed that molybdate (MoVI) was first reduced to MoV by molybdenum reductase prior to the joining of phosphate anions forming molybdenum blue [8, 9].

To date, about 25 molybdenum-reducing bacteria from 8 different genera (Acinetobacter, Bacillus, Burkholderia, Enterobacter, Escherichia, Klebsiella, Pseudomonas and Serratia) were reported to be isolated from different soils and polluted waters around the world. However, report on the isolation of molybdenum-reducing bacteria from Nigerian soil is scarce, making it necessary to conduct this research. This work characterizes the potential of a locally isolated Pantoea sp. strain HMY-P4 to reduce toxic hexavalent molybdenum to insoluble molybdenum blue.

**MATERIALS AND METHODS**

**Chemicals and equipment**
All chemical reagents and media ingredients used in this research were of analytical grade. The glassware used for the experiment were washed with 10% (v/v) nitric acid and rinsed with double distilled water to avoid the effect of other metals contaminants. Following oven drying at 60 °C, glassware was sterilized by autoclaving at 121 °C, 115 kPa for 15 min. Glucose was separately autoclaved and added to the medium prior to autoclaving at 121 °C, 115 kPa for 15 min. Glucose was separately autoclaved and added to the medium afterwards. The molten medium was allowed to cool to about 50 °C before pouring onto sterile disposable petri dishes. The plates were then kept at 30 °C in the incubator for overnight drying. In case of prolong storage, tape was used to seal and prevent the plates from drying out [12].

**Low phosphate-molybdate (LPM) agar**
This medium was prepared by dissolving agar: 18 g, (NH4)2SO4: 3 g, MgSO4.7H2O: 0.5 g, NaCl: 5 g, Na2MoO4.2H2O: 2.42 g, Na2HPO4: 0.71 g, yeast extract: 0.5 g and glucose: 10 g into a liter of deionized water and adjusting the pH to 7.5 prior to autoclaving at 121 °C, 115 kPa for 15 min. Glucose was separately autoclaved and added to the medium afterwards. The resultant 1,050 bases were blast using NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/) to compare with the GenBank database. Blast analysis revealed that the sequence is related to the family Enterobacteriaceae. The 16S ribosomal RNA gene sequence of this isolate will be deposited in the GenBank.

**Isolation of Mo-reducing bacteria**
Soil sample was collected in March 2018 from agricultural land in Ungogo latitude 12.087077 and longitude 8.480645 Kano state, Nigeria. The sample was collected 5 cm beneath the top soil using a sterile spatula, transferred into suitable container and stored in refrigerator at 4 °C before use. After a careful serial dilution in sterile distilled water, an aliquot (0.1 ml) was spread-plated on LPM agar and incubated at 37 °C for 24 h. Molybdate reduction was determined by inoculating 2% (v/v) of the selected isolate (OD600 = 0.9-1.0) into 100 ml of freshly prepared low phosphate molybdate media and incubated at 25 °C for 24 h. The intensity of the molybdenum blue produced was measured at 685 nm. The isolate with the highest intensity of Mo-blue was selected for further study.

**16s rRNA Gene Sequencing**
A single colony of the best molybdenum-reducing bacterium grown on nutrient agar was suspended in 1 ml of distilled water, and extracted the genomic DNA by alkaline lysis using Commercially prepared genomic DNA purification kit (Thermo Scientific). The forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-AAGGAGGTGATCCAGCGGA-3') primers were used to amplify the 16S rRNA. PCR amplification was performed by initial denaturation at 94 °C for 3 min, 25 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min, then a final extension at 72 °C for 10 min using Gradient touch thermo-cycler [16].

**Phylogenetic analysis**
The phylogenetic analysis was performed using clustal W by multiple alignments of twenty retrieved 16S rRNA gene sequences (from GenBank) that closely matched isolate D through PHYLIP output option. All possible missed-alignments were manually checked, and gaps were excluded from the computation. The phylogenetic tree was constructed using PHYLIP version 3.573, with Serratia marcescens as the out-group in the phylogram.

The evolutionary distance matrices for the neighbor-joining/ UPGMA method were computed through DNADIST algorithm program, while nucleotide substitution was performed using Jukes and Cantor model. The confidence levels of individual tree branches were checked by repeating the PHYLIP analysis with 1,000 bootstraps. Majority rule (50%) consensus trees were constructed for the topologies found using a family of consensus tree methods using the CONSENSE program and the tree was viewed using Tree View [12].
Effect of electron donor sources on molybdate reduction

The effect of various electron donors such as glucose, fructose, sucrose, starch and glycerol on molybdenum reduction by this bacterium was determined by supplementing 1% (w/v) into LPM containing fixed concentrations of phosphate (5 mM) and sodium molybdate (10 mM) at pH 7.0. Into freshly prepared LPM, a 2% (v/v) of bacterial aliquot in nutrient broth (OD600=0.9-1.0) was inoculated, the culture media were then incubated for 24 h at 25 °C. After the required incubation, an aliquot (3 ml) from the culture media was transferred into centrifuging tube and centrifuged at 4,000 rpm for 10 min. The resultant supernatant was spectrophotometrically measured at 865 nm for the amount of Mo-blue produced [12].

Effect of glucose concentrations

Following screening of the best electron donor source, glucose was found to be the best electron donor source that supports optimum Mo-blue production by this bacterium and thus analyzed for optimum concentration. In the present study, the effect of various concentrations of sucrose (0-50 g/L) supplemented into LPM were tested. After 24 h incubation, an aliquot (3 ml) from the culture media was centrifuged at 4,000 rpm for 10 min at room temperature. The resultant supernatant was spectrophotometrically measured at 865 nm for the amount of Mo-blue produced as before.

Screening of nitrogen source

Apart from a carbon source, the nitrogen source is also an absolute requirement for bacterial growth. Similarly, a balance in C:N ratio is important as this can influence the reduction process. Thus, it is necessary to screen for the suitable nitrogen sources that support molybdenum reduction in this bacterium. In this study, the effect of both organic and inorganic nitrogen sources like ammonium sulfate, phenyl alanine, urea, glutamate and glycine on molybdenum reduction was determined by supplementing 0.3% (w/v) each into LPM containing fixed concentrations of phosphate (5 mM) and sodium molybdate (10 mM) at pH 7.0. After 24 h incubation, an aliquot (3 ml) from the culture media was centrifuged at 4,000 rpm for 10 min at room temperature. The resultant supernatant was measured spectrophotometrically at 865 nm for the amount of Mo-blue produced.

Effect of initial pH of LPM

In this study, the effect of initial pH of the medium was used to determine the suitable pH that supports optimum Mo-blue production in this bacterium. The buffer system used was the disodium phosphate in LPM, which span the pH range between 5.5 and 8.0 (pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0). After 24 h incubation, an aliquot (3 ml) from the culture media centrifuge at 4,000 rpm for 10 min. The resultant supernatant was then measured spectrophotometrically at 865 nm for the amount of Mo-blue produced.

Effect of temperature

The effect of temperature was examined over a temperature range (25—50 °C) was used to evaluate the effect of temperature. After 24 h incubation, an aliquot (3 ml) from the culture media was centrifuged at 4,000 rpm for 10 min at room temperature. The resultant supernatant was then read spectrophotometrically at 865 nm for the amount of Mo-blue produced.

Effect of phosphate and molybdate concentrations

Phosphate and molybdate concentrations are major parameters influencing microbial molybdate reduction to Mo-blue. In this study, the effect of phosphate concentration was evaluated by fixing molybdate concentration as 10 mM and varying the phosphate concentrations (1.5, 2.9, 3.5, 5.0, 7.5 mM). Similarly, the effect of molybdate concentration was evaluated by fixing phosphate concentration at 5 mM and varied the molybdate concentrations (10-100 mM). After the required incubation, an aliquot (3 ml) from the culture media was centrifuged at 4,000 rpm for 10 min at room temperature. The resultant supernatant was then read spectrophotometrically at 865 nm for the amount of Mo-blue produced.

Statistical Analysis

One-way analysis of variance was performed using a statistical software INSTAT GraphPad version 3.0.

RESULTS

Identification of Molybdenum-Reducing Bacterium

The bacterial isolate was screened for its capacity to reduce molybdate to Mo-blue. The colony morphology on nutrient agar showed a smooth, circular, while Gram’s stain microscopic observation revealed that the bacterium is Gram-negative, rod-shaped. Phylogenetic analysis of the 16S rRNA gene sequence (Fig. 1) using the neighbor-joining method revealed a bootstrap value of 75% similarity to Pantoea agglomerans strain LMG 1286 indicating that the phylogenetic relationship of this isolate to a particular species will be difficult. Thus, the isolate was tentatively assigned as Pantoea sp. strain HMY-P4.

![Phylogram (neighbor-joining method)](image)

**Fig. 1.** Phylogram (neighbor-joining method) indicating the genetic relationship between isolate A and referenced related microorganisms based on 16S rRNA gene sequence analysis. Accession numbers are accompanied by the species names of their 16S rRNA sequences. The numbers at branching points or nodes refer to bootstrap values, based on 1000 re-samplings. Scale bar represents 100 nucleotide substitutions. Serratia marcescens is the out-group.

Effect of electron donor sources on molybdate reduction

Different electron donor sources such as glucose, sucrose, fructose, starch and lactose were used at an initial concentration of 1% (w/v) to study their effects on the molybdate reduction efficiency of the bacterium. The bacterium utilizes all the carbon sources. However, glucose was the best carbon source (Fig. 2). There was no significant difference between sucrose and fructose. The least utilized carbon was starch. The best glucose concentration was at 5 mM (Fig. 3).
Fig. 2. Effect of various electron donor sources on molybdenum reduction by *Pantoea* sp. after 24 h incubation at the final concentration of 1% (w/v). Data represent mean ± standard deviation of triplicate. Values with different letters over the bars are significantly different (p<0.05).

Fig. 3. Effect of different glucose concentrations on molybdenum reduction by *Pantoea* sp. after 24 h incubation. Data represent mean ± standard deviation of triplicate.

**Effect of nitrogen source on molybdate reduction**

Different nitrogen sources such as ammonium sulphate, glycine, phenylalanine, glutamic acid and urea were used to study the effect of nitrogen source on the bacterium. Fig. 4, shows that ammonium sulphate was the best followed by glycine and then phenylalanine, glutamate and the least was urea.

Fig. 4. Effect of various nitrogen sources on molybdenum reduction by *Pantoea* sp. after 24 h incubation at the final concentration of 0.3% (w/v). Data represent mean ± standard deviation of triplicate. Values with different letters over the bars are significantly different (p<0.05).

**Effect of pH and temperature on molybdate reduction**

The effect of temperature on molybdate reduction of bacterium was studied at temperature ranging from 25, 30, 35, 40, and 50. The result shows that the optimum temperature is between 35 and 40 °C. After 45 °C, the bacteria growth dropped rapidly and almost no growth occurred at temperature 50 °C and above (Fig. 5). High level of Mo-blue complex production was between pH 6 and 8. The pH of the medium affected the reduction of molybdenum. Most of the best pH for best reduction of the bacteria is ranging between pH 6 and 8 (Fig. 6).

Fig. 5. Effect of different pH on molybdenum reduction by *Pantoea* sp. after 24 h incubation. Data represent mean ± standard deviation of triplicate.
Effect of phosphate and molybdate concentrations on Mo-blue production

The study of the effect of molybdate concentrations on molybdate reduction was carried out at 10, 20, 40, 60, 80, and 100 mM respectively. Fig. 7 shows that molybdate reduction was found to increase linearly as molybdate concentration was increased from 10 mM attaining optimum at 40 mM, while molybdate reduction was inhibited at higher concentrations. Phosphate concentration for molybdate reduction was set at 1.5, 2.9, 3.5, 5.0, and 7.5 mM, and 5 mM was found to be optimum in this bacterium (Fig. 8).

Study on the effect of various parameters such as source of electron donors, temperature, molybdate, and phosphate on molybdate reduction is important. This knowledge not only is important for contributing to the fundamental understanding of the mechanism of reduction but also will be beneficial in the area of bioremediation of molybdenum especially with pH and source of electron donors as these parameters can be controlled by addition of suitable compounds into the soil. Glucose appears to be the main carbon source supporting Mo-blue production in this bacterium. In contrast, sucrose was the best source of electron donor for E. coli 48, S. marcescens strain Dr.Y6, and Serratia sp. strain Dr.Y5 [9,13,20]. Fructose was the best carbon source in strain hkeem [21].

It was previously demonstrated in E coli 48 that molybdate reduction is growth-associated and this is also true for several other Mo-reducing bacteria [13]. Hence, it is not surprising that glucose supported the highest reduction as it is the best substrate for growth and source of carbon as well as the best substrate for producing reducing equivalents in the form of NADH or NADP. However, in terms of cost effectiveness, sucrose in the form of cane and sugar molasses is preferred to pure and simple carbohydrates as it is a cheaper alternative found in abundance in industrial wastes [21]. Thus, sucrose in the form of molasses would be employed for future molybdenum bioremediation using this bacterium.

Campbell et al. [7] were the first to note the inhibitory effects of elevated concentrations of both phosphate and molybdate ions on bacterial Mo-blue production. Hence, it is very important to ascertain the effects of phosphate and...
molybdate ions on molybdate reduction in this bacterium. A similar ratio of phosphate to molybdate was seen in E. coli K12 and Klebsiella sp. strain Hkeem [21], where, at 5 mM phosphate, 40 mM molybdate was the optimum concentration for supporting Mo-blue production [7]. In Ec 48, the optimum ratio is 5 mM phosphate to 20 mM molybdate.

In all of the Mo-reducing bacteria studied so far, phosphate concentrations higher than 5 mM inhibited molybdate reduction. Phosphate disrupts the phosphomolybdate complex preventing reduction to Mo-blue [22]. The highest reported concentration of molybdenum as a pollutant is at 2000 ppm (20.8 mM molybdate) from a molybdenum mine runoff [23]. At this concentration, all of the bacteria studied so far can reduce molybdate provided that the soil phosphate concentrations do not exceed 20 mM for appreciable reduction to take place. Fortunately, phosphate soil concentrations rarely exceeded this value [9].

The study of temperature optimal for the growth of microbes would be very useful for bioremediation and maximizing enzyme yield for purification purposes. Pantoea sp. isolated shows optimal temperature between 35 and 40 ºC. This correlate with the bacterium Pseudomonas sp. strain DRY2 [15], which shows maximum temperature at 40 ºC. However, in contrast to E. coli K12 which reduces molybdenum optimally between 30 and 36 ºC [7]. Ghani et al. [9] reported 30 ºC as the optimum. Other Mo-reducing bacteria isolated so far have optimal temperature between 30 and 37 ºC.

Although, it is not generally possible to change temperature when performing onsite bioremediation, screening for indigenous microbes for local bioremediation works is the norm since these microbes would have an optimum temperature close to the temperature of the site chosen for bioremediation. The optimal initial pH supporting reduction is also shared by majority of the Mo-reducing bacteria [7, 9, 13, 20, 21, 24]. The obligation for neutral pH and a moderate temperature range ensures that bioremediation treatments will be economical, and these observable facts are also shared by many bacteria. However, most soils with active metabolic activity usually exhibited lowering in pH due to several factors such as carbohydrate fermentation and carbon dioxide production leading to the lowering of pH [25].

CONCLUSION

A locally isolated bacterium with potential to reduce toxic hexavalent molybdenum to precipitable Mo-blue was isolated and characterized. The bacterium was tentatively identified as Pantoea sp. strain HMY-P4 based on the 16S rRNA partial sequencing and molecular phylogenetic analysis. Glucose, an easy assimilable carbon source was the best electron donor at 5 mM phosphate. Phosphate disrupts the phosphomolybdate complex preventing reduction to Mo-blue [22]. The highest reported concentration of molybdenum as a pollutant is at 2000 ppm (20.8 mM molybdate) from a molybdenum mine runoff [23]. At this concentration, all of the bacteria studied so far can reduce molybdate provided that the soil phosphate concentrations do not exceed 20 mM for appreciable reduction to take place. Fortunately, phosphate soil concentrations rarely exceeded this value [9].

The study of temperature optimal for the growth of microbes would be very useful for bioremediation and maximizing enzyme yield for purification purposes. Pantoea sp. isolated shows optimal temperature between 35 and 40 ºC. This correlate with the bacterium Pseudomonas sp. strain DRY2 [15], which shows maximum temperature at 40 ºC. However, in contrast to E. coli K12 which reduces molybdenum optimally between 30 and 36 ºC [7]. Ghani et al. [9] reported 30 ºC as the optimum. Other Mo-reducing bacteria isolated so far have optimal temperature between 30 and 37 ºC.

Although, it is not generally possible to change temperature when performing onsite bioremediation, screening for indigenous microbes for local bioremediation works is the norm since these microbes would have an optimum temperature close to the temperature of the site chosen for bioremediation. The optimal initial pH supporting reduction is also shared by majority of the Mo-reducing bacteria [7, 9, 13, 20, 21, 24]. The obligation for neutral pH and a moderate temperature range ensures that bioremediation treatments will be economical, and these observable facts are also shared by many bacteria. However, most soils with active metabolic activity usually exhibited lowering in pH due to several factors such as carbohydrate fermentation and carbon dioxide production leading to the lowering of pH [25].

CONCLUSION

A locally isolated bacterium with potential to reduce toxic hexavalent molybdenum to precipitable Mo-blue was isolated and characterized. The bacterium was tentatively identified as Pantoea sp. strain HMY-P4 based on the 16S rRNA partial sequencing and molecular phylogenetic analysis. Glucose, an easy assimilable carbon source was the best electron donor at 5 g/L. Also, ammonium sulphate was the best nitrogen source. Other requirements include a strict phosphate concentration at 5.0 mM, molybdate concentration between 20 and 40 mM, pH between 6 and 8 and temperature at 35 and 40 ºC. This bacterium could be suitable for bioremediation in temperate regions like Nigeria.

ACKNOWLEDGMENT

We thank the Department of Biochemistry Bayero University Kano, Nigeria funding this work.

