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Soluble Molybdenum Reduction by *Morganella* sp. Locally-isolated from Agricultural Land in Kano

Mohammed, S.[1], Gafasa, M.A.[1], Kabara, H.T.[1], Babandi, A.[1], Shehu, D.[1], Ya'u, M.[1], Abubakar, S.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,

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ABSTRACT

Intensive agriculture and industrial activities have significantly increased the global burden of pollutants; thus, bioremediation of these pollutants is intensely sought. A bacterium with potential of reducing toxic soluble molybdenum to precipitable molybdenum blue (Mo-blue) was isolated from agricultural soil in Darmanawa, Kano state. The bacterium grown on low phosphate media (LPM) reduces molybadate to Mo-blue optimally at pH between 6.0 and 7.5, temperature of 35 °C, glucose was the best electron donor source at 5 g/L and ammonium sulphate was the best nitrogen source. The optimum molybdate concentration supporting the reduction process was 40 mM at 3.5 mM phosphate. Phylogenetic analysis of 16S rRNA partial sequence identified the bacterium as *Morganella* sp. The ability of this isolate to reduce toxic soluble molybdenum to colloidal less toxic form is novel and makes the bacterium an important instrument for bioremediation of this pollutant.

INTRODUCTION

Environmental pollution keeps on increasing at an alarming rate due to certain activities of man such as rapid industrialization, unsafe agricultural practices, technological advancement, and urbanization which degrades the environment. Heavy metals liberated into the environment are persistent due to their toxicity which constitutes a serious menace to organisms exposed to high levels of such pollutants. Metals are essential to the biological functions of plants and animals but at high levels, they interfere with metabolic reactions in systems of organisms. Toxic heavy metals such as molybdenum (Mo), lead (Pb), cadmium (Cd), mercury (Hg), chromium (Cr), zinc (Zn), uranium (Ur), selenium (Se), silver (Ag), gold (Au), nickel (Ni) and arsenic (As) which are not useful to plants, are capable of reducing plant growth due to reduced photosynthetic activities, plant mineral nutrition, and reduced activity of essential enzymes [1,2].

Heavy metals are cytotoxic at low concentrations and could lead to cancer in humans [3]. These toxic metals could accumulate in the body when consumed in contaminated food through the food chain and become health risks to living organisms [4]. This causes oxidative stress, an irregularity involving the production of free radicals and the capacity of cells to annihilate them or repair the damage [5,6]. This leads to base damage through formation of reactive oxygen species (ROS) which includes oxygen radicals (superoxide and hydroxyl) [7] and non-radical derivatives of molecular oxygen (O_2) such as hydrogen peroxide (H_2O_2), as well as breakage of the DNA molecule [5,6]. Heavy metal toxicity increases the production of reactive oxygen species (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.

A relatively recent technology to remediate heavy metal pollution is bioremediation. Bioremediation is an option that offers the possibility to destroy or render harmless various contaminants using natural biological activity. As such, it uses relatively low-cost, low-technology techniques, which generally have a high public acceptance and can often be carried out on site. Bioremediation has been used at a number of sites worldwide, including Europe, with varying degrees of success. Techniques are improving as greater knowledge and experience are gained, and there is no doubt that bioremediation has great potential for dealing with certain types of site pollution. Molybdenum is one of the heavy metals of which its pollution is

reported globally [8]. For example, the sea of the Tokyo Bay in Japan is contaminated with molybdenum reaching up to several hundreds of ppm [8]. Molybdenum is relatively low in toxicity to human, but it is known to be very toxic to ruminant animals. The scouring in cows was reported after grazing in areas contaminated with molybdenum at up to 5 ppm [9]. The technology utilizes microorganisms from a variety of genera. Metals that could be detoxified via biological reduction include mercury, chromium, molybdenum, arsenic, lead, copper, uranium, selenium, bismuth, and tungsten [10].

Studies on microbial molybdenum reduction as a potential molybdenum bioremediation are important because molybdenum pollution is an emerging global pollutant. In Tyrol, Austria, molybdenum pollution is caused by industrial emissions and has contaminated large pasture areas, reaching as high as 200 ppm and causing scouring in ruminants [11]. Molybdenum is a component of agricultural fertilizer, therefore, wash off agricultural lands results in polluting our water bodies with this metal, hence, requiring for its remediation.
In this study, the potential of a locally isolated *Morganella* sp. to reduce soluble molybdenum to a precipitable molybdenum blue (Mo-blue) is reported.

MATERIALS AND METHODS

Culture media preparation
All media preparations (solid and broth) were made according to the recipe of Ghani *et al.* [12] and Shukor *et al.* [18] except otherwise stated.

Low phosphate-molybdate (LPM) agar
This medium was prepared by dissolving agar: 18 g, $(NH4)_2SO_4$: 3 g, $MgSO_4.7H_2O$: 0.5 g, NaCl: 5 g, $Na_2MoO_4.2H_2O$: 2.42g, Na_2HPO_4: 0.71g, 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 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 [16].

Low phosphate-molybdate medium (LPM)
This medium was prepared according to the method of Ghani *et al.* [14] with slight modification. Briefly, into a liter of deionized water, $(NH4)_2SO_4$: 3 g, $MgSO_4.7H_2O$: 0.5 g, NaCl: 5 g, $Na_2MoO_4.2H_2O$: 2.42 g, Na_2HPO_4: 0.71 g, yeast extract: 0.5 g and glucose: 10 g were dissolved and the medium was adjusted to pH 7.5 prior to autoclaving at 121 °C, 115 kPa for 15 min. Glucose was separately autoclaved and added to the medium afterwards.

Isolation of Mo-reducing bacteria
Soil sample was collected in June 2018 from agricultural land in Darmanawa, Tarauni LGA (latitude 11.9613047 and longitude 8.5458373 and) 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 possible Mo-blue production. According to Campbell *et al.* [17] bacterial molybdate reduction produces Mo-blue on a low phosphate molybdate medium. Therefore, colony that forms highest blue color intensity was isolated and re-streaked on low phosphate molybdate agar to obtain a pure culture. Following 24 h incubation on nutrient agar, a single isolated colony was inoculated into a fresh nutrient broth and incubated at 30 °C for 24 h. Molybdate reduction was determined by inoculating 2% (v/v) of the selected isolate (OD_{600} = 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 865 nm. The isolate with the highest intensity of Mo-blue was selected for further study.

Identification of Mo-reducing bacteria

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 the genomic DNA was extracted by alkaline lysis using Commercially prepared genomic DNA purification kit (Thermo Scientific). The forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-AAGGAGGTGATCCAGCCGCA-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].

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.

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 *Pantoea agglomerans* 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 [16].

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 (OD_{600}= 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 cetrifuging tube and centrifuged at 4,000 rpm for 10 min. The resultant supernatant was then measured spectrophotometrically at 865 nm for the amount of Mo-blue produced [16].

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 then measured spectrophotometrically at 865 nm for the amount of Mo-blue produced as before.

Screening of nitrogen source

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 centrifuge 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 ^{o}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 AND DSICUSSION

The bacterial reduction of molybdenum to molybdenum blue was initially reported more than a century ago in 1896 [18]. In the last century, isolation of Mo-reducing bacteria was reported as early as 1939 [19]. After a long absence, it was reported again in 1985 [17] and in 1993 [14]. Ghani *et al*. (1993) [14] were the first to quickly recognize the potential of molybdenum-reducing bacterium for the bioremediation of molybdenum.

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 low bootstrap value of less than 50% similarity to *Morganella* sp. indicating that the phylogenetic relationship of this isolate to a particular species will be difficult. Thus, the isolate was tentatively assigned as *Morganella* sp.

The vast majority of the molybdenum-reducing bacteria reported to date are heterotrophs belonging to the *Enterobacteriaceae* family [14, 20-22] with the exception of *Acidothiobacillus thiooxidans* [23] and several *Bacillus* spp. [24-28]. The heterotrophic nature of this family of bacteria enables them to ferment simple sugars resulting in lowering the pH of the growth medium and thus inducing the formation of phosphomolybdate, a vital intermediate in the reduction of molybdate to Mo-blue [29].

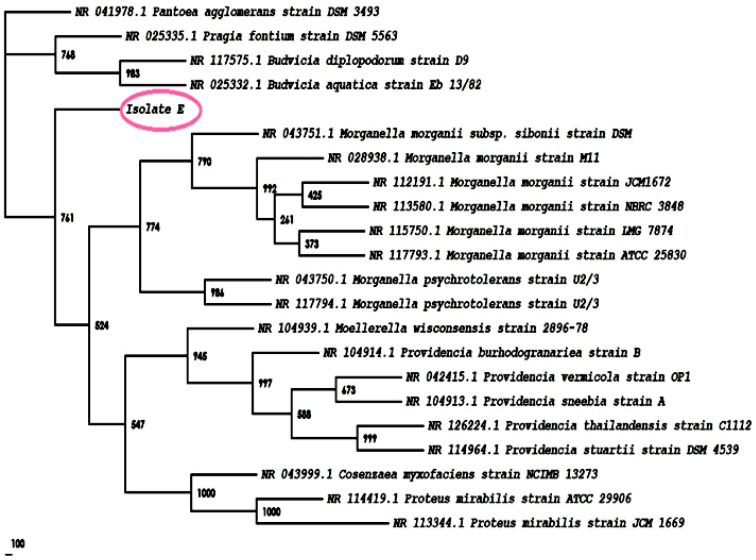

Fig. 1. Phylogram (neighbor-joining method) indicating the genetic relationship between isolate E and referenced related microorganisms based on 16S rRNA gene sequence analysis. Accession numbers are accompanied by the species names of their 16S rRNA sequences. The numbers at branching points or nodes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

Effect of electron donor sources on molybdate reduction

Different carbon sources such as glycerol, sucrose, glucose, fructose and starch were used at an initial concentration of 1.0% (w/v) in low phosphate media to ascertain the effect of electron donor sources. The result shows that after 24 hours of incubation, glucose was the best electron donor source followed by sucrose, glycerol, fructose and starch, respectively (**Fig. 2**). This result corresponds with the work of Shukor *et al.* [30]. Nearly all the Mo-reducing bacteria isolated to date prefer either glucose or sucrose as best source electron donor for Mo-blue production, with *Klebsiella oxytoca* strain hkeem being the only bacterium that prefers fructose.

One of the reasons why simple carbohydrates such as sucrose and glucose are the preferred electron donors is that they can easily produce reducing equivalents NADH and NADPH through metabolic pathways like glycolysis, Krebs's cycle and electron transport chain. Both reducing equivalents (NADH and NADPH) are substrates for the molybdenum reducing-enzyme [31,20]. Nevertheless, when it comes to bioremediation, affordability is the most important factors, and a cheaper carbon source may be needed for an economic process.

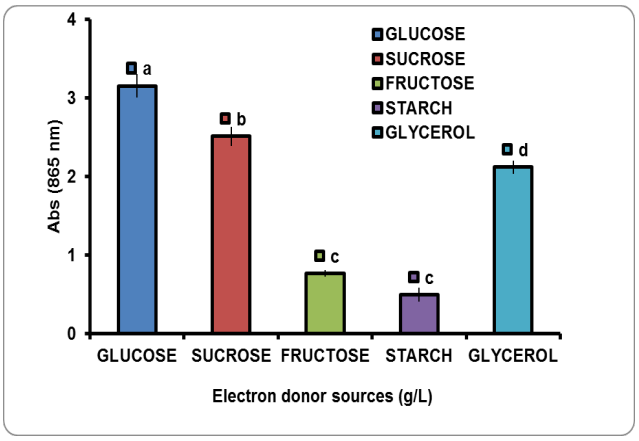

Fig. 2. Effect of various electron donor sources on molybdenum reduction by *Pseudomonas* sp. after 24 h incubation at the final concentration of 1% (w/v). Data represent mean ± standard deviation of triplicate.

Effect of glucose concentration

The effect of various glucose concentrations was determined to know the optimal and maximal concentration affecting molybdenum reduction. The result shows that 5 g/L is the optimal concentration supporting Mo-blue production in this bacterium with no significant difference ($p>0.05$) between 10 and 40 g/L, following 24 h incubation at 25 °C (**Fig. 3**). Higher glucose concentrations above 40 g/L or 4% was found to inhibit molybdenum reduction.

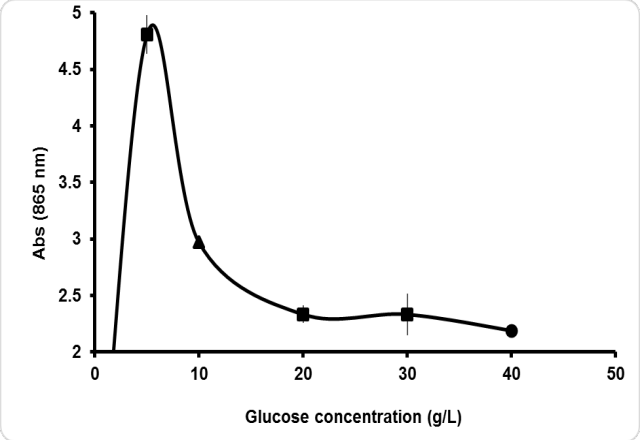

Fig. 3. Effect of different glucose concentrations on molybdenum reduction by *Morganella* sp. after 24 h incubation. Data represent mean ± standard deviation of triplicate.

Effect of nitrogen source on molybdate reduction

The effect of nitrogen source on molybdate reduction was determined using various nitrogen sources such as ammonium sulphate, glycine, urea, phenylalanine and glutamic acid. After 24 hours of incubation, ammonium sulphate was found be the best nitrogen source, followed by glycine, urea, phenyl alanine and glutamic acid, respectively, with no significant difference ($p>0.05$) between glutamate and phenyl alanine (**Fig. 4**). However, after 48 hours of incubation, ammonium sulfate was found to have the most intense blue color, thus ammonium sulphate was chosen as nitrogen source for subsequent analysis. Moreover, ammonium sulphate was best nitrogen source for almost all the molybdenum-reducing bacteria isolated to date.

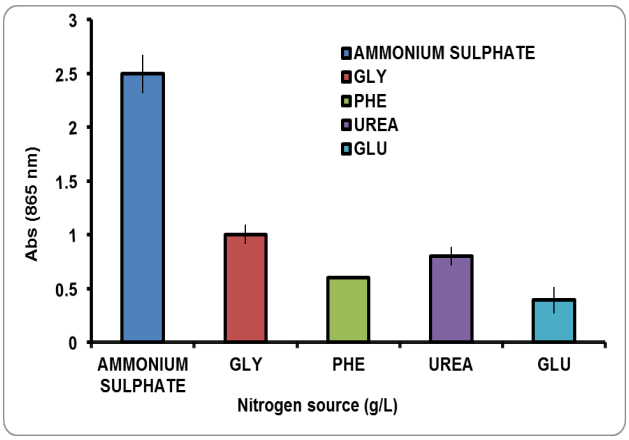

Fig. 4: Effect of various nitrogen sources on molybdenum reduction by *Morganella* sp. after 24 h incubation at the final concentration of 0.3% (w/v). Data represent mean ± standard deviation of triplicate. Values with different letters over the bars are significantly different ($p<0.05$).

Effect of pH on molybdate reduction

The effect of pH on molybdenum reduction in this bacterium was evaluated at different initial pH range, 5.0 - 7.5. The result shows that optimum Mo-blue production was found around a wide pH range between 6.0 and 7.5 (**Fig. 5**). Molybdenum reduction was not supported at pH below 5.0 possibly due to inhibition of the bacterial growth. Previous studies have shown that optimum Mo-blue production occurred at pH between 6.0 and 7.0 in *Pseudomonas* sp. strain DRY2, *Enterobacter* sp. strain Dr.Y13 and *Klebsiella oxytoca* strain hkeem, which is in agreement with findings of this research. pH is a measure of acidity, alkalinity or neutrality of a medium. Microorganisms, like all other living organisms, prefer a physiological pH to survive and carry out metabolic processes. Their ability to survive higher or lower pH depends on their capacity to regulate the pH difference of the intracellular and extracellular environment [32].

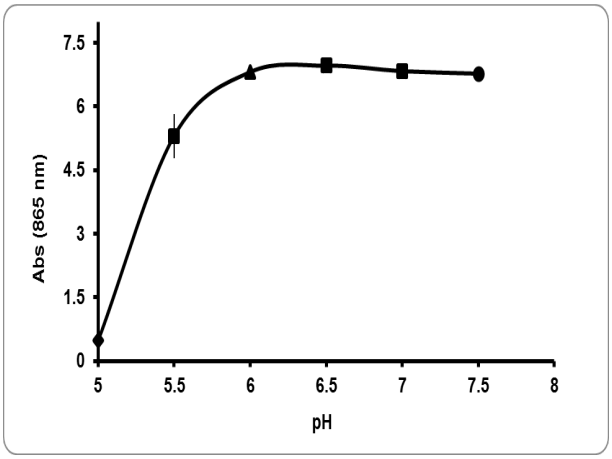

Fig. 5. Effect of different pH on molybdenum reduction by *Morganella* sp. after 24 h incubation. Data represent mean ± standard deviation of triplicate.

Effect of temperature on molybdenum reduction

The effect of temperature on molybdenum reduction was determined over a temperature range of 25 - 50 °C). It was found that the optimum temperature supporting molybdate reduction in this bacterium is 35 °C, a significant decrease ($p<0.05$) in Mo-blue production was observed at a temperature higher than 40 °C (**Fig. 6**). Interestingly, this finding is in line with the previous works, since the optimum temperature supporting molybdate reduction in all isolated Mo-reducing bacteria to date is between 30 and 40 °C, except for *Pseudomonas* sp. Dr.Y1 with optimal temperature between 15 and 20 °C, possibly because it was isolated from Antarctica. Temperature is the measure of the degree of hotness or coldness of a medium. Since metabolic activity in the microorganisms involves enzyme proteins, which are liable to irreversible denaturation at a temperature above or below optimum. It is, therefore, necessary to ascertain the desirability and tolerance of the bacterium for effective bioremediation.

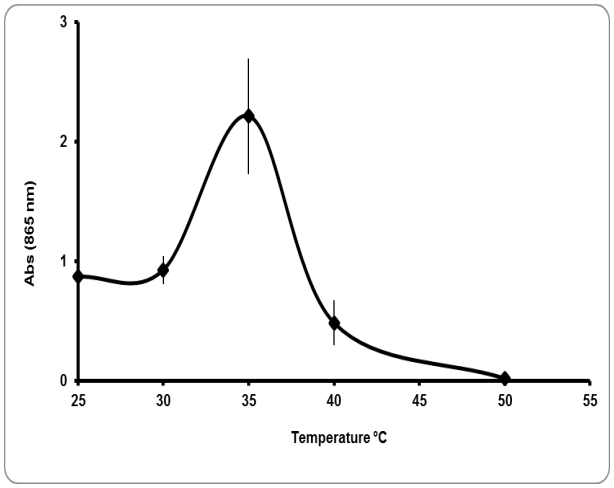

Fig. 6. Effect of different temperature on molybdenum reduction by *Morganella* sp. after 24 h incubation. Data represent mean ± standard deviation of triplicate.

Effect of phosphate and molybdate concentrations on Mo-blue production

Determining the effect of phosphate and molybdate concentrations supporting optimal molybdenum reduction is important because both anions have been shown to inhibit Mo-blue production in bacteria [33]. The effect of molybdate concentration on the reduction process was ascertained using various concentrations ranging between 10-100 mM at fixed phosphate concentration. Mo-blue production in this bacterium was found to be optimal over a wide range of sodium molybdate concentration (40 mM) (**Fig. 7**). Previously isolated Mo-reducing bacteria showed optimal reduction at concentrations between 5 to 80 mM (450 to 7,600 mg/L). The highest concentration of molybdenum found in the environment is from an abandoned uranium mine in Colorado with concentration reaching 6,550 mg/Kg [34]. On the other hand, the concentrations of phosphate required for optimal Mo-blue production is 3.5 mM (**Fig. 8**). This finding is agreeing with previous works that show a very narrow phosphate concentration of 2.9 - 5.0 mM, with higher concentrations strongly inhibiting the reduction process. A phosphate concentration of 100 mM ceased Mo-blue production. Several of the Mo-reducing bacteria isolated can be utilized for the bioremediation of molybdenum pollution due to their high tolerance towards molybdenum.

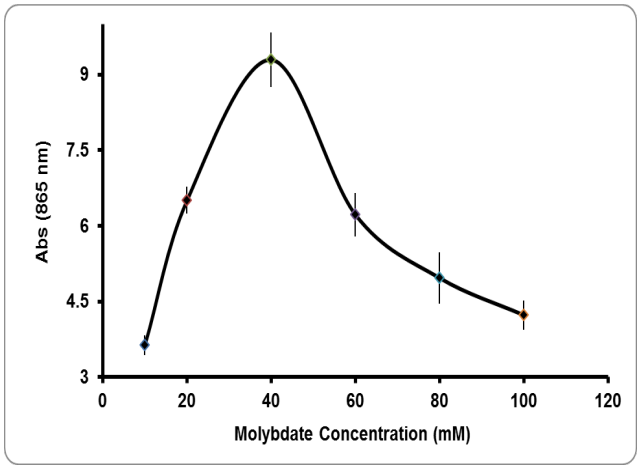

Fig. 7. Effect of molybdate concentration on Mo-blue production by *Morganella* sp. after 24 h incubation at fixed phosphate concentration. Data represent mean ± standard deviation of triplicate.

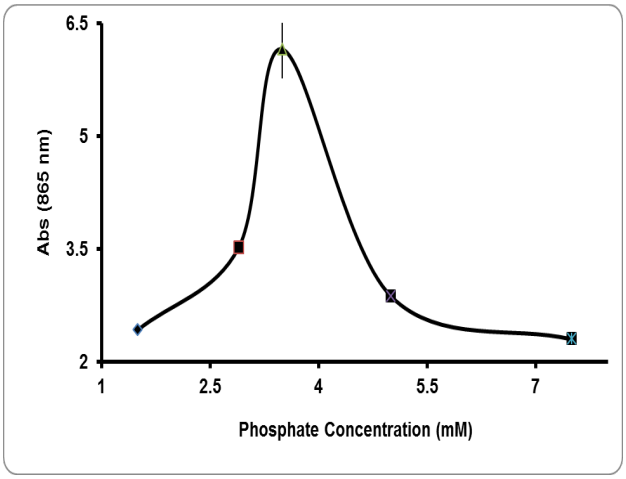

Fig. 8. Effect of phosphate concentrations on molybdenum reduction by *Morganella* sp. after 24 h incubation at fixed molybdate concentration. Data represent mean ± standard deviation of triplicate.

CONCLUSION

A bacterium with ability to reduce hexavalent molybdenum to insoluble Mo-blue has been successfully isolated from agricultural soil in Darmanawa Tarauni LGA Kano. Microscopic examination reveals the isolate as Gram-negative rod-shaped, which is identified as *Morganella* sp. based on phylogenetic analysis of 16S rRNA partial sequencing. The isolate reduces molybdenum optimally at 5 g/L glucose, pH between 6.0 and 7.5, temperature between 35 ^{o}C, molybdate and phosphate concentrations at 40 mM and 3.5 mM respectively. The isolate utilizes glucose and ammonium sulphate as best carbon and nitrogen sources, respectively. This finding will be helpful in future bioremediation of molybdenum using this isolate.

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