

Isolation and Characterization of Molybdenum-reducing *Pseudomonas* sp. from Agricultural Land in Northwest-Nigeria

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ABSTRACT

Microbial heavy metals reduction has an important role in biological system and in environmental metals recycling to remove its toxic effects in soil and wastewater. A molybdenum-reducing bacterium with potential to reduce toxic hexavalent molybdenum to colloidal molybdenum blue (Mo-blue) was isolated from agricultural land in Northwest-Nigeria. The Gram's stain and microscopic examination reveals that the isolate is a Gram-negative, however, phylogenetic analysis of the 16S rRNA identifies the isolate as *Pseudomonas* sp. Molybdenum reduction in this bacterium is optimally supported by glucose at 1.0% (w/v). The optimum phosphate and molybdate concentrations supporting molybdate reduction were between 3.5 and 7.5 mM for phosphate and 100 mM molybdate. Molybdate reduction is optimum at 37 °C and pH between 6.5 and 7.5. The capacity of this isolate to reduce toxic molybdenum to a less toxic form is novel and makes the bacterium important instrument for bioremediation of this pollutant.

INTRODUCTION

Pollution is reduction in the quality of the environment by introduction of impurities. Smoke and dust contaminate air; junks etc. Polluted land and industrial wastes, municipal sewage and domestic waste cause water pollution. Most of the industries release their waste directly (without any treatment) into the stream, lakes, oceans as well as in the open land and that contaminate the ground water and soils [1]. Food chain contamination by heavy metals has become a burning issue in recent years because of their potential accumulation in bio-systems through contaminated water, soil and air. Therefore, a better understanding of heavy metal sources, their accumulation in the soil and the effect of their presence in water and soil on plant systems seem to be particularly important matter of present-day research on threat assessments [2].

Molybdenum though an essential heavy metal, at elevated levels, it is toxic to ruminants. The wide application of molybdenum industry has resulted in several soil and water pollution cases all around the world such as in the Tokyo Bay, Tyrol in Austria, the black sea [3]. There are numbers of contaminants like fertilizers, pesticides, heavy metals which seriously affect human by entering the system directly or

indirectly through food material. Pollution of environment with heavy metals is a crucial problem as it causes toxicity. 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 [4]. Therefore, there is need to remove or reduce this element from our environment. Molybdenum is fast becoming a global pollutant. Its pollution ranging from hundreds to thousands part per million (ppm) has been documented in water and soils all around the world. Molybdenum toxicity in inhibiting spermatogenesis and arresting embryogenesis in organisms, such as catfish and mice, at levels as low as several parts per million have been reported [5,6,7,8]. Perhaps, molybdenum at levels as low as ppm is highly toxic to ruminants, especially cows [9].

In Antarctica, due to anthropogenic activities, molybdenum has been found as pollutant even in the absence of mining activities (Santos *et al.*, 2005). Molybdenum toxicity differs according to the route of ingestion. The absorption rate following oral ingestion depends on the compounds solubility and the diet composition [7]. Similarly, the chemical form in which molybdenum exist greatly influence the rate of its bioavailability and depends on the animal species [10]. In ruminants, however, concentrations of 10 mg/kg of body weight molybdenum resulted

in tissue copper depletion, potentiated by dietary sulfate [11]. 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 [7]. In a similar report, Halmi and Ahmad [11], 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 count.

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 afterward 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.

Culture media preparation

All media preparations (solid and broth) were made according to the recipe of Ghani *et al.* [12] and Shukor *et al.* [13] except otherwise stated.

Low phosphate-molybdate (LPM) agar

This medium was prepared by dissolving agar: 18 g, (NH₄SO₄): 3 g, MgSO₄·7H₂O: 0.5 g, NaCl: 5 g, Na₂MoO₄·2H₂O: 2.42g, Na₂HPO₄: 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 [14].

Low phosphate-molybdate medium (LPM)

This medium was prepared according to the method of Ghani *et al.* [12] with slight modification. Briefly, into a liter of deionized water, (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 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 from agricultural land in Hoto (longitude:11.5889059 and latitude:8.1529749), Baure, Kano State-Nigeria in March 2018. The sample was collected 5 cm beneath the topsoil 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.* [15] 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 (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 865 nm. The isolate with the highest intensity of Mo-blue was selected for further study.

Identification of Mo-reducing bacteria

The morphological characteristics of the isolate were observed on LPM agar, nutrient agar and by Gram staining. The molecular phylogenetic analysis was used to identify the isolate at the genus level [10].

Gram stain

This experiment classifies bacteria either as Gram positive or Gram negative. The former usually retains the purple primary stain colour, while the later absorb the counter stain and become pink or red. A smear from fresh culture (18 h) was prepared and flooded with crystal violet and allowed for one min, then gently washed with distilled water. There follows the addition of Gram's iodine, ethyl alcohol (95%) and safranin respectively, each allowed for a min before washing. The slides were finally examined under a microscope.

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 [14]. 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 A 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 *Pseudomonas* 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 [14].

Effect of different 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 then measured spectrophotometrically at 865 nm for Mo-blue produced [14].

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 Mo-blue produced as before.

Nitrogen source screening

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 sodiummolybdate (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 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 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 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, 7.5mM). 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 Mo-blue produced.

RESULTS and DISCUSSION

The bacterial reduction of molybdenum to molybdenum blue was initially reported more than a century ago in 1896 [16]. In the last century, isolation of Mo-reducing bacteria was reported as early as 1939 (Jan 1939). After a long absence, it was reported again in 1985 [15] and in 1993 [12]. Ghani *et al.* [12] 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 *Pseudomonas protegens* indicating that the phylogenetic relationship of this isolate to any species will be difficult. Thus, the isolate was tentatively assigned as *pseudomonas* sp. Majority of the molybdenum-reducing bacteria reported to date are heterotrophs belonging to the Enterobacteriaceae family [12,17,18,19] with the exception of *Acidithiobacillus thiooxidans* [20] and several *Bacillus* spp [21,22,23,24]. 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 [25].

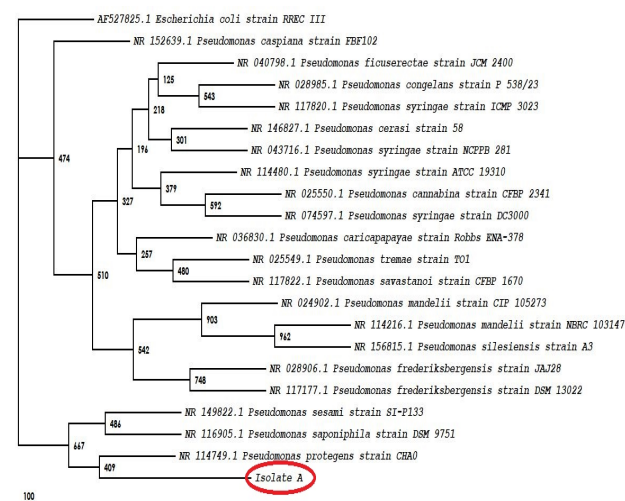


Fig. 1. Phylogram (neighbor-joining method) indicating the genetic relationship between isolate A and referenced related microorganisms based on 16S rRNA gene sequence analysis. Accession numbers are accompanied by the species names of their 16S rRNA sequences. The numbers at branching points or nodes refer to bootstrap values, based on 1000 re-samplings. Scale bar represents 100 nucleotide substitutions. *E. coli* 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, fructose, starch and glycerol, respectively (**Fig. 2**). This result corresponds with the work of Gafasa *et al.* [26]. 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 [27]. Nevertheless, when it comes to bioremediation, affordability is the most important factors, and a cheaper carbon source may be needed for an economic process.

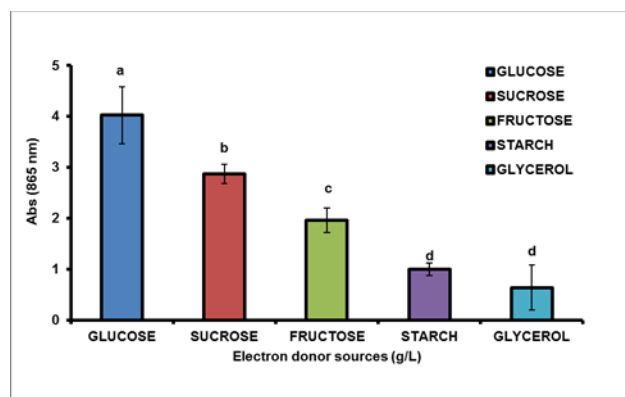


Fig. 2. Effect of various electron donor sources on molybdenum reduction by *Pseudomonas* sp. after 24 h incubation at the final concentration of 1% (w/v). Data represent mean \pm standard deviation of triplicate. Values with different letters over the bars are significantly different ($p < 0.05$).

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.

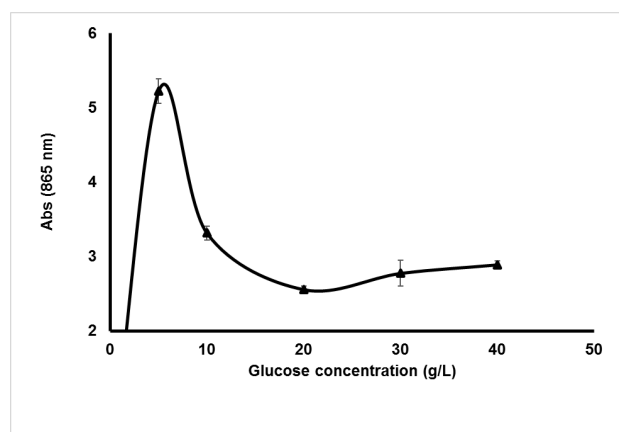


Fig. 3. Effect of different glucose concentrations on molybdenum reduction by *Pseudomonas* sp. after 24 h incubation. Data represent mean \pm standard deviation of triplicate.

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, glutamate, urea and phenylalanine. After 24 hours of incubation, glycine was found to be the best nitrogen source, followed by ammonium sulphate, glutamate, phenyl alanine and urea, 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.

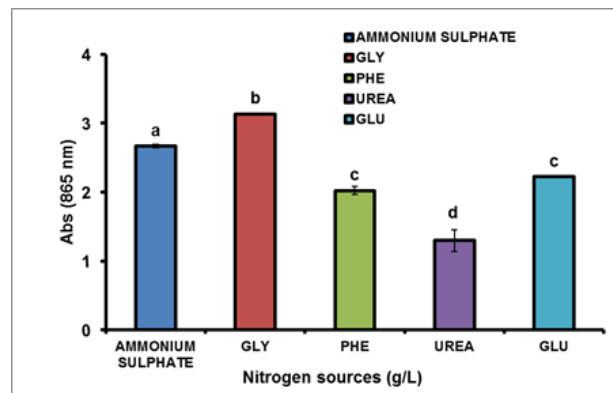


Fig. 4. Effect of various nitrogen sources on molybdenum reduction by *Pseudomonas* sp. after 24 h incubation at the final concentration of 0.3% (w/v). Data represent mean \pm standard deviation of triplicate. Values with different letters over the bars are significantly different ($p < 0.05$).

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 - 8.0. The result shows that optimum Mo-blue production was found around a wide pH range between 6.5 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 and 7 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 [28].

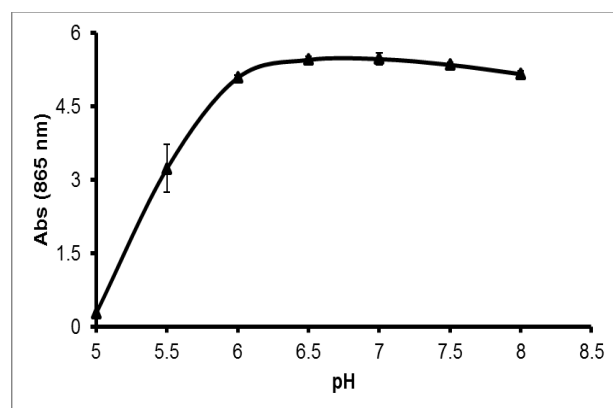


Fig. 5. Effect of different pH on molybdenum reduction by *Pseudomonas* sp. after 24 h incubation. Data represent mean \pm standard deviation of triplicate.

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 between 35 and 40 °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.

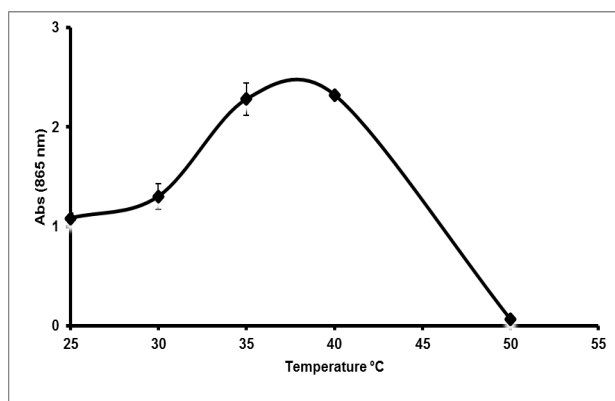


Fig. 6. Effect of different temperature on molybdenum reduction by *Pseudomonas* sp. after 24 h incubation. Data represent mean \pm standard deviation of triplicate.

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 [29]. 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 (between 60 and 100 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 concentrations of molybdenum found in the environment is from an abandoned uranium mine in Colorado with concentration reaching 6,550 mg/Kg [30]. On the other hand, the concentrations of phosphate required for optimal Mo-blue production ranged between 3.5 and 7.5 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.

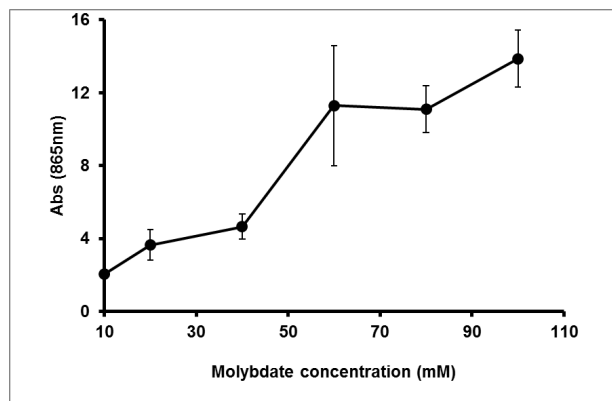


Fig. 7. Effect of molybdate concentration on Mo-blue production by *Pseudomonas* sp. after 24 h incubation at fixed phosphate concentration. Data represent mean \pm standard deviation of triplicate.

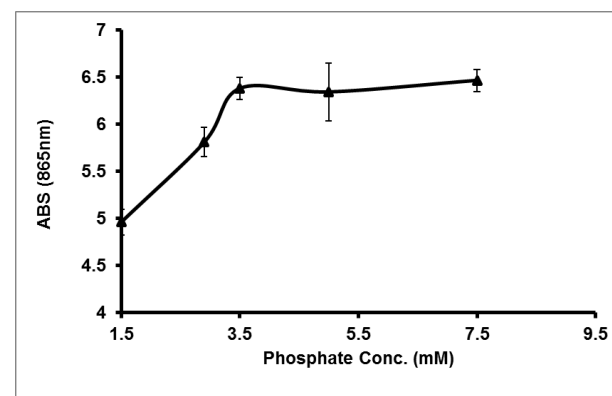


Fig. 8. Effect of phosphate concentrations on molybdenum reduction by *Pseudomonas* sp. after 24 h incubation at fixed molybdate concentration. Data represent mean \pm standard deviation of triplicate.

CONCLUSION

A Mo-reducing bacterium has been successfully isolated from agricultural soil. The Gram-negative *Bacillus* with ability to reduce molybdenum optimally at 5 g/L glucose, pH between 6.5 and 7.5, temperature between 35 and 40 °C, 60 to 100 mM molybdate and phosphate concentration between 3.5 and 7.5 mM. The isolate utilizes glucose and ammonium sulphate as best carbon and nitrogen sources, respectively, and the isolate is tentatively identified as *Pseudomonas* sp. based on phylogenetic analysis of 16S rRNA partial sequencing. This finding will be helpful in bioremediation.

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