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Characterizing the Molybdenum-reducing Properties of *Pseudomonas* sp. locally isolated from Agricultural soil in Kano Metropolis Nigeria

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ABSTRACT

Pollution of the environment by heavy metals and other toxic xenobiotics has increasingly become global public health concern. Bacterial reduction of molybdenum to insoluble molybdenum blue (Mo-blue) forms the basis for its bioremediation. A bacterium with the ability to reduce toxic soluble molybdenum has been isolated from Agricultural soil and identified as *Pseudomonas* sp. based on the 16S rRNA partial sequencing and phylogenetic analysis. Spectroscopic analysis reveals that the bacterium reduced sodium molybdate to Mo-blue optimally at pH between 6.5 and 7.0, temperatures between 35 °C and 40 °C. Glucose was the best electron donor source supporting molybdate reduction, followed by sucrose, fructose, glycerol and starch in descending order. Other requirements include a phosphate concentration of 3.5 mM and a molybdate concentration of between 40 and 60 mM. The absorption spectrum of the Mo-blue produced was similar to the previously isolated Mo-reducing bacteria and closely resembles a reduced phosphomolybdate.

INTRODUCTION

Pollution of the environment by hazardous waste materials including organic pollutants and heavy metals, has adversely affected the natural ecosystem to the detriment of man. These pollutants arise from anthropogenic sources as well as natural disasters such as hurricanes and volcanic eruptions. Toxic metals could accumulate in agricultural soils and get into the food chain, thereby becoming a major threat to food security [1].

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 [1]. Toxic heavy metals such as 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]

Molybdenum has also been known to cause significant sewage sludge pollution that poses a health hazard [3] Several reports have been made by researchers regarding molybdenum toxicity in inhibiting spermatogenesis and arresting embryogenesis in organisms such as catfish and mice at levels as low as several parts per million. Cows are the best example of the most affected ruminants, with scouring and deaths observed at levels as low as several parts per million [4, 5] Molybdenum is as toxic as other heavy metals such as chromium and lead [6].

Molybdenum reduction by microbe is an interesting phenomenon because the product shows an intense blue color allowing the progress of reduction to be observed. Molybdate reductions by microbes have been reported since the last 100 years [7, 8], however, it was not until 1985 that *Campbell et al.* revitalizes the phenomenon of molybdate reduction in *Escherichia coli* K12 [9]. Initially, it was 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 [10]. However, this mechanism of molybdate reduction is not plausible taking into account the chemistry of molybdenum. A new mechanism of molybdate reduction in EC 48 was proposed involving phosphomolydate as an intermediate between molybdate and molybdenum blue. This mechanism was proposed because the spectrum of molybdenum blue from EC 48 closely matches that of molybdenum blue from the phosphate determination method which is known to be a reduced phosphomolybdate. In 1988, Sugio *et al.* reported on the reduction of molybdate into molybdenum blue by *Thiobacillus ferreoxidans* strain AP19–3

[10]. The reducing activity was initially only attributed to the enzyme sulphur/ferric ion oxidoreductase (SFORase). However, further studies showed that the Fe^{2+} present in the media could chemically reduce molybdate to Mo-blue under acidic conditions aside from enzymatic reduction [11]. In 1993, Ghani *et al.* reported that a heterotrophic bacterium, *Enterobacter cloacae* strain 48 (EC 48), could also reduce molybdate to molybdenum blue. The molybdenum blue product adheres strongly to cells indicating the potential removal of the metal from solution as bioremediation tool [12]. The scarcity of the report on molybdate reduction is reflected in the citation absence of the works of Campbell *et al.* [9] by Sugio *et al.* [10] and Ghani *et al.* [12]. For the past 25 years, studies on microbial molybdate reduction to molybdenum blue were carried out in *E. coli* and another local bacterium EC 48 [12-14].

Despite the fact that details on the bioreduction of molybdenum exists almost three decades ago, but there are scanty researches on molybdenum bioremediation from Nigeria and in Africa at large [15-17].

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.* [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 [19].

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, $(NH4)_2SO_4$: 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 from Agricultural land in Guringawa, Kumbotso Local Govt. Area, Kano State-Nigeria in March 2018. 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.* [9] 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

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 [19].

Gram stain

This experiment classifies bacteria either as Gram positive or Gram negative. The former usually retains the purple primary stain color, 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 [19]. 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 [19].

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 ^{o}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 [19].

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
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 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 DISCUSSION

The bacterial reduction of molybdenum to molybdenum blue was initially reported more than a century ago in 1896 [7]. In the last century, isolation of Mo-reducing bacteria was reported as early as 1939. After a long absence, it was reported again in 1985 [9] and in 1993 [12]. Ghani *et al*. were the first to quickly recognize the potential of molybdenum-reducing bacterium for the bioremediation of molybdenum [12].

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 a particular species will be difficult. Thus, the isolate was tentatively assigned as *Pseudomonas* sp. The vast majority of the molybdenum-reducing bacteria reported to date are heterotrophs belonging to the Enterobacteriaceae family) [12; 20-22] with the exception of *Acidothiobacillus thiooxidans* [10] and several *Bacillus* spp [23-25]. 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 [26].

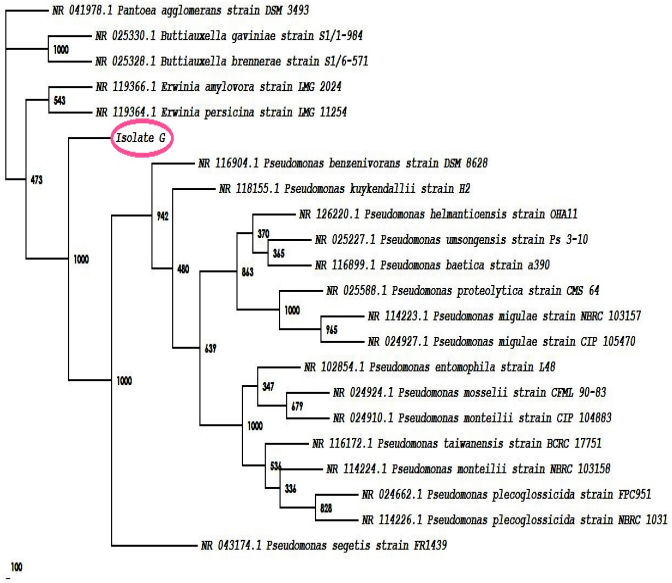

Fig. 1. Phylogram (neighbor-joining method) indicating the genetic relationship between isolate A and referenced related microorganisms based on 16S rRNA gene sequence analysis. Accession numbers are accompanied by the species names of their 16S rRNA sequences. The numbers at branching points or nodes refer to bootstrap values, based on 1000 re-samplings. Scale bar represents 100 nucleotide substitutions. *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, fructose, starch and glycerol, respectively (**Fig. 2**). This result corresponds with the work of Shukor *et al.* [27]. 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 [20, 28]. Nevertheless, when it comes to bioremediation, affordability is the most important factors, and a cheaper carbon source may be needed for an economic process.

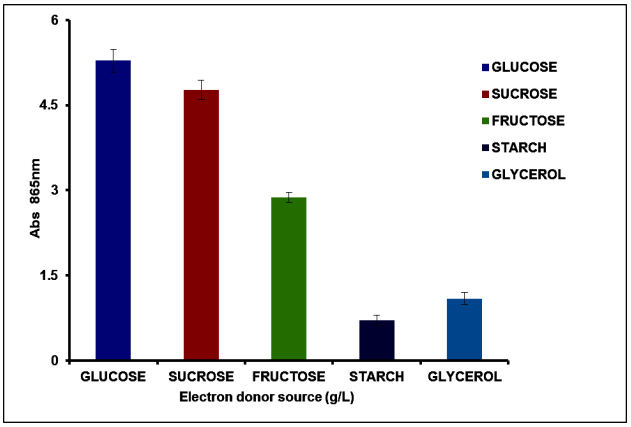

Fig. 2. Effect of various electron donor sources on molybdenum reduction by *Pseudomonas* sp. after 24 h incubation at the final concentration of 1% (w/v). Data represent mean ± standard deviation of triplicate.

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.

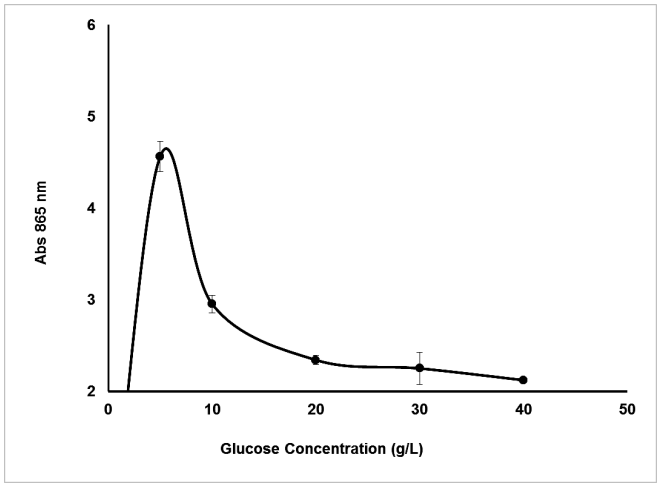

Fig. 3. Effect of different glucose concentrations on molybdenum reduction by *Pseudomonas* sp. after 24 h incubation. Data represent mean ± standard deviation of triplicate.

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

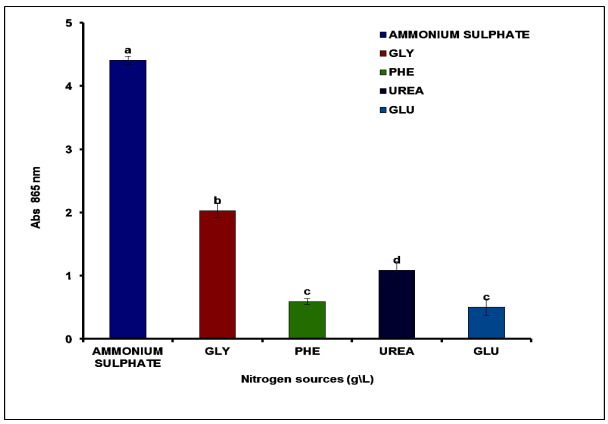

Fig. 4. Effect of various nitrogen sources on molybdenum reduction by *Pseudomonas* sp. after 24 h incubation at the final concentration of 0.3% (w/v). Data represent mean ± standard deviation of triplicate. Values with different letters over the bars are significantly different ($p<0.05$).

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 7.0 (**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 [29].

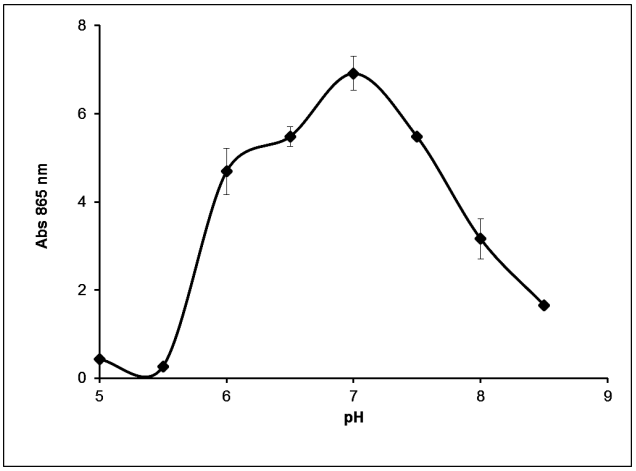

Fig. 5. Effect of different pH on molybdenum reduction by *Pseudomonas* sp. after 24 h incubation. Data represent mean ± standard deviation of triplicate.

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 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 ºC, 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.

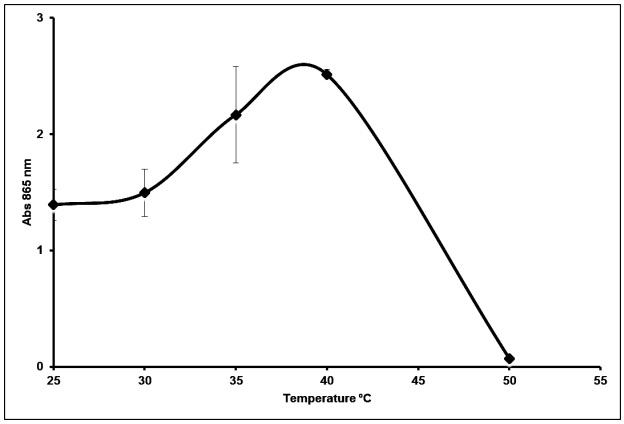

Fig. 6. Effect of different temperature on molybdenum reduction by *Pseudomonas* sp. after 24 h incubation. Data represent mean ± standard deviation of triplicate.

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 [30]. 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 40 and 60 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 [31]. 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 to 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.

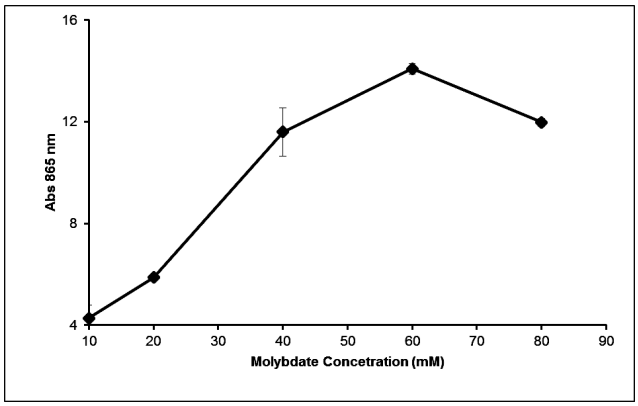

Fig. 7. Effect of molybdate concentration on Mo-blue production by *Pseudomonas* sp. after 24 h incubation at fixed phosphate concentration. Data represent mean ± standard deviation of triplicate.

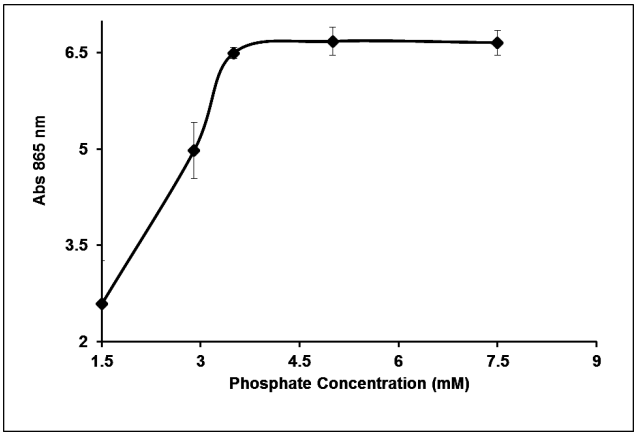

Fig. 8. Effect of phosphate concentrations on molybdenum reduction by *Pseudomonas* sp. after 24 h incubation at fixed molybdate concentration. Data represent mean ± standard deviation of triplicate.

CONCLUSION

A Mo-reducing bacterium has been successfully isolated from Agricultural soil in Kano metropolis, Nigeria. Microscopic examination reveals that the isolate is Gram-negative *Bacillus* with ability to reduce molybdenum optimally at 5 g/L glucose, pH between 6.5 and 7.0, temperature between 35 and 40 °C, 40 - 60 mM sodium molybdate and phosphate concentration of 3.5 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 forms the fundamental use of this isolate in future bioremediation particularly from Africa region. Research is underway to isolate and purify the enzyme responsible molybdate reduction form this bacterium.

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REFERENCES

1. Ojuederie OB, & Babalola OO. Microbial and Plant-Assisted Bioremediation of Heavy Metal Polluted Environments: A Review. Int. J Env. Res. 2017: 26. https://doi.org/10.3390/ijerph14121504.
2. Nematian MA & Kazemeini F. Accumulation of Pb, Zn, C and Fe in plants and hyperaccumulator choice in galali iron mine area, Iran. Int. J. Agric. Crop Sci. 2013: 5, 426.
3. Neunhäuserer C, Berreck M & Insam, H. Remediation of soils contaminated with molybdenum using soil amendments and phytoremediation. Water Air Soil Poll. 2001: 128(12):85–96.
4. Yagamuchi S, Miura C, Ito A, Agusa T, Iwata H, Tanabe S & Miura, T. Effects of lead, molybdenum, rubidium, arsenic and organochlorines on spermatogenesis in fish: Monitoring at Mekong Delta area and in vitro experiment. Aqua Toxicol. 2007, 83(1): 43-46
5. Zhang YL, Liu FJ, Chen XL, Zhang ZQ, Shu RZ & Yu, X.L. Dual effects of molybdenum on mouse oocyte quality and ovarian oxidative stress. Syst Biol Reprod Med. 2013: 59(6):312–318.
6. Kincaid RL. Toxicity of ammonium molybdate added to drinking water of calves. J Dairy Sci.1980: 63(4):608–10.
7. Capaldi A, Proskauer B. Beiträge Zur Kenntniss Der Säurebildung Bei Typhus-Bacillen Und Bacterium Coli - Eine Differential-Diagnostische Studie, Zeitschrift für Hygiene und Infectionskrankheiten.1896: 23*:* 452-474;
8. Bautista EM, Alexander M. Reduction of inorganic compounds by soil microorganisms. Soil Sci Soc Am Proc. 1972;36:918–20.
9. Campbell AM, Del Campillo-Campbell A, Villaret DB. Molybdate reduction by *Escherichia coli* K-12 and its chl mutants. Proc Natl Acad Sci U S A. 1985;82(1):227–31.
10. Sugio T, Tsujita Y, Katagiri T, Inagaki K & Tano T. (1988). Reduction of Mo^{6+} with elemental sulfur by *Thiobacillus ferrooxidans*. J Bacteriol, 1988: 170(12), 5956–5959.
11. Yong NK, Oshima M, Blake RC & Sugio T. Isolation and some properties of an iron-oxidizing bacterium *Thiobacillus ferrooxidans* resistant to molybdenum ion. Biosci Biotech Biochem. 1997 **61**, 1523–1526.
12. Ghani B, Takai M, Hisham NZ, Kishimoto N, Ismail, AKM, Tano T & Sugio, T. Isolation and characterization of a Mo^{6+}-reducing bacterium. J Basic Microbiol. 1993: **59**(4):1176–1180.
13. Ariff AB, Rosfarizan M, Ghani B, Sugio T & Karim MIA. World J Microbiol Biotechnol.1997: **13**, 643–647.
14. Shukor MY, Shamaan NA, Syed MA, Lee CH, Karim MI. Characterization and quantification of molybdenum blue production in *Enterobacter cloacae* strain 48 using 12-molybdophosphate as the reference compound. Asia-Pacific J Mol Biol Biotechnol. 2000: 8(2):167-72.
15. Shukor MY, Othman M, Karamba KI, Halmi MIE, Rahman MF, Yasid NA, Ahmad SA & Yakasai HM. Isolation and characterization of Molybdenum-reducing and PEG-degrading *Enterobacter cloacae* strain KIK-14 in Agricultural soil from Nigeria. J Environ Microbial Toxicol. 2017: 5(1): 4-11.
16. Karamba KI & Yakasai HM. Isolation and Characterization of a Molybdenum-reducing and Methylene Blue-decolorizing *Serratia marcescens* strain KIK-1 in soils from Nigeria. Biorem Sci Technol Res. 2018: 6(1): 1-8
17. Maarof MZ, Shukor MY, Othman M, Karamba KI, Halmi MIE, Rahman MF & Yakasai HM. Isolation and Characterization of a Molybdenum-reducing *Bacillus amyloliquefaciens* strain KIK-12 in soils from Nigeria with the Ability to grow on SDS. J Environ Microbial Toxicol. 2018: 6(1): 13-20
18. Shukor MY, Lee CH, Omar I, Karim MIA, Syed MA & Shamaan N A. Isolation and characterization of a molybdenum-reducing enzyme in *Enterobacter cloacae* strain 48. Pertanika J Sci Technol. 2003: 11(2), **261–272**.
19. Yakasai HM. (2017). Microbial molybdate reduction to Mo-blue by a cyanide-degrading bacterium. Thesis submitted to the school of Graduate studies, Universiti Putra Malaysia, in fulfilment of the requirements for the degree of Doctor of Philosophy.
20. Shukor MY, Habib SH, Rahman, MF, Jirangon H, Abdullah MP, Shamaan NA & Syed MA. Hexavalent molybdenum reduction to molybdenum blue by *S. marcescens* strain Dr. Y6. Appl Biochem Biotechnol. 2008: 149(1):33– 43. doi: 10.1007/s12010-008-8137-z.
21. Shukor MY, Rahman MFA, Suhaili Z, Mustafa S, Shamaan NA, Syed MA Reduction of Mo(VI) by the bacterium *Serratia* sp. Strain DRY5. J Environ Biol. 2009:30(1): 65-72.
22. Shukor MY, Ahmad SA, Nadzir MMN, Abdullah MP, Shamaan NA, Syed MA. Molybdate reduction by *Pseudomonas* sp. Strain DRY2. J Appl Microbiol. 2010:108(6):2050-8.
23. Abo-shakeer LKA, Ahmad SA, Shukor MY, Shamaan NA & Syed NA. Isolation and characterization of a molybdenum-reducing *Bacillus pumilus* strain lbna. J Environ Microbiol Toxicol. 2013; 1(1):9-14.
24. Othman AR, Bakar NA, Halmi MIE, Johari WLW, Ahmad SA, Jirangon H & Shukor MY. Kinetics of molybdenum reduction to molybdenum blue by *Bacillus* sp. strain A.rzi. Biomed Res Int. 2013: 28(1):68–76.
25. Halmi MIE, Wasoh H, Sukor S, Ahmad SA, Yusof MT & Shukor MY. Bioremoval of molybdenum from aqueous solution. Int J Agric Biol. 2014: 16: 848–850.
26. Khayat ME, Rahman MFA & Shukor MS. Characterization of a molybdenum reducing *Bacillus* sp. strain Khayat with the ability to grow on SDS and diesel. Rendiconti Lincei. 2016: 29,547-556.
27. Shukor Y, Adam H, Ithnin K, Yunus I, Shamaan NA & Syed A. Molybdate reduction to molybdenum blue in microbe proceeds via a phosphomolybdate intermediate. J Biol Sci. 2007: **7**(8):1448–52.
28. Shukor MS & Shukor MY. A microplate format for characterizing the growth of molybdenum reducing bacteria. J Environ Microbiol Toxicol. 2014: 2(2):1–3.
29. Shukor MY, Rahman, MF, Suhaili Z, Shamaan NA & Syed MA. Reduction of molybdenum blue by *Serratia marcescens* strain DRY9. Pol J Microbiol. 2009: 58(2):141-147.

30. Sabullah MK, Rahman MF, Ahmed SA, Sulaiman MR, Shukor MS & Shamaan NA. Isolation and characterization of a molybdenum-reducing and glyphosphate-degrading *Klebsiella oxytoca* strain saw-5 in soils from Sarawak. Agrivita. 2016: 38(1):1-13.
31. Stone J & Stetler L. Environmental Impacts from the North Cave Hills Abandoned Uranium Mines, South Dakota in: Uranium, Mining and Hydrogeology (Editors: Merkel, B., Hasche- Berger, A.), Springer Berlin Heidelberg, 2008: 371-380, http://dx.doi.org/10.1007/978-3-540- 87746-2_48;