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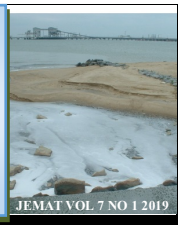

Isolation and Characterization of Molybdate-reducing *Enterobacter cloacae* from Agricultural Soil in Gwale LGA Kano State, Nigeria

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ABSTRACT

Agricultural and industrial activities contribute most to the pollutants found globally; therefore, bioremediation of these pollutants is intensely sought. This research isolated a molybdenum-reducing bacterium from agricultural soil. The bacterium grown in low phosphate media (LPM) reduces molybadate to Mo-blue optimally at pH between 6.5 and 7.0, temperature between 35 and 40 °C, glucose at 5 g/L and glycine at 3 g/L were the best electron donor and nitrogen sources, respectively. The optimum molybdate concentration is between 80 and 100 mM, and phosphate concentration was between 5.0 and 7.5 mM. Phylogenetic analysis of 16S rRNA partial sequencing identified the bacterium as *Enterobacter cloacae*. The capacity of this bacterium to reduce toxic molybdenum to less toxic colloidal molybdenum blue is novel and form the basis for its use in future bioremediation of this pollutant.

INTRODUCTION

The environment is made up of three components namely: air, soil and water. According to [1] quality of life on Earth is linked inextricably to the overall quality of the environment. The problems associated with polluted environment now assume increasing prominence in many countries. Contaminated water bodies and lands generally result from past industrial activities when awareness of the health and environmental effects connected with the production, use, and disposal of hazardous substances were less well recognized than today. Environmental pollution is globally, and the estimated number of polluted environments is significant and its continual discovery over recent years has led to international efforts to remedy many of these environments, either as a response to the risk of adverse health or environmental effects caused by pollution or to enable the area to be redeveloped or restored for use. [2].

Bioremediation involved the use of biological systems for the removal or reduction of contamination from air, soil and water. The process involves the use of organism obtained from the environment of interest or imported from other system and exposing it to a target contaminant so as to reduce or remove the toxic component [3-7]. Therefore, bioremediation is the application of bio systems such as microbes, plants and animals to reduce the potential toxicity of any contaminants in the environment by degrading, transforming and immobilizing these undesirable substances to less harmful forms Successful [5,8] bioremediation is dependent on an interdisciplinary approach involving such disciplines as microbiology, engineering, ecology, geology, and chemistry [9].

However, bioremediation research and practice are currently still hampered by an incomplete understanding of the genetics and genome-level characteristics of the organisms used, the metabolic pathways involved, and their kinetics. The result of this is an inability to model and predict the behavior of these processes, and hence a difficulty in developing natural bioremediation processes in the field. Bioremediation, which involves the use of microbes to detoxify and degrade environmental contaminants, has received increasing attention in recent times to clean up a polluted environment [10]. Besides the environmentally friendly properties of the bioremediation it is also cost effective compared to other techniques, which use expensive chemicals, consume high amount of energy or require expensive technology. Before implementation of bioremediation, the- 1 -microbiological processes need to be well understood to avoid side effects such as degradation of chemicals to some toxic or harmful mobile substances [11].

Microbial bioreduction and bio-precipitation of heavy metals is one of such bioremediation technologies to remove these contaminants from the environment. The first report on bacterial reduction of toxic molybdenum is more than a century old. However, report on this phenomenon is lacking from Nigeria, despite the large quantity of metal pollution generated in the country. This work reported the isolation and characterization of a novel molybdate-reducing *Enterobacter cloacae* from Agricultural soil in Gwale LGA Kano state, Nigeria.

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.* [13] except otherwise stated.

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 in 2018 from agricultural land in Dorayi Gwale LGA (longitude 11.691304 and latitude 6.4573), Kano State-Nigeria. The sample was collected 5 cm below 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. 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 ºC 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 ºC for 3 min, 25 cycles at 94 ºC for 1 min, 50 ºC for 1 min, and 72 ºC for 2 min, then a final extension at 72 ºC 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 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 *Enterobacter cloacae.* 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 carbon sources

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 ºC. After the required incubation, an aliquot (3 ml) from the culture media was centrifuged at 4,000 rpm for 10 min. The resultant supernatant was spectrophotometrically read at 865 nm for the amount of Mo-blue produced [14].

Effect of glucose concentrations

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.

Screening of nitrogen source

Nitrogen source is also an absolute requirement for bacterial growth. 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

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. 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 ºC) 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

Molybdate and phosphate 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

Data are expressed as mean ± SD of triplicate using INSTAT GraphPad version 3.0 statistical software.

RESULTS AND DSICUSSION

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. 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 *Enterobacter hormaechei* indicating that the phylogenetic relationship of this isolate to a particular species will be difficult. Thus, the isolate was tentatively assigned as *Enterobacter clocae*.

The vast majority of the molybdenum-reducing bacteria reported to date are heterotrophs belonging to the Enterobacteriaceae family [12,17-19] with the exception of *Acidothiobacillus thiooxidans* [20] and several *Bacillus* spp. [21-24] 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].

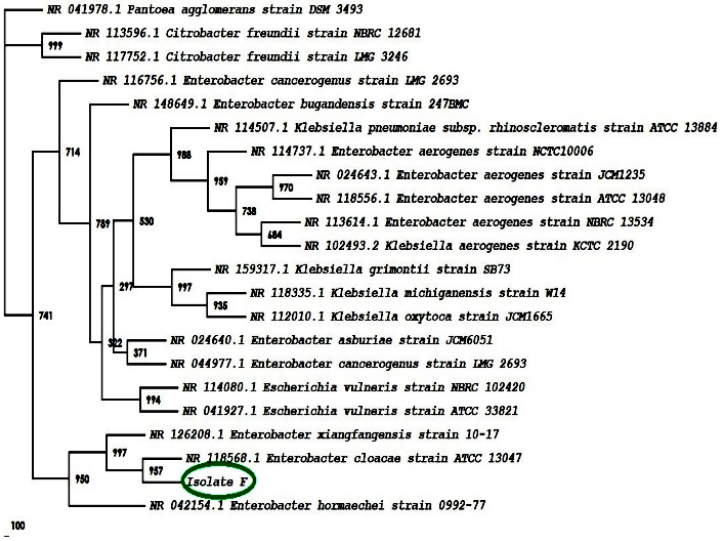

Fig. 1: Phylogram (neighbor-joining method) indicating the genetic relationship between isolate F and referenced related microorganisms based on 16S rRNA gene sequence analysis. Accession numbers are accompanied by the species names of their 16S rRNA sequences. The numbers at branching points or nodes 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

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 h 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.* [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 [17]. Nevertheless, when it comes to bioremediation, affordability is the most important factors, and a cheaper carbon source may be needed for an economic process.

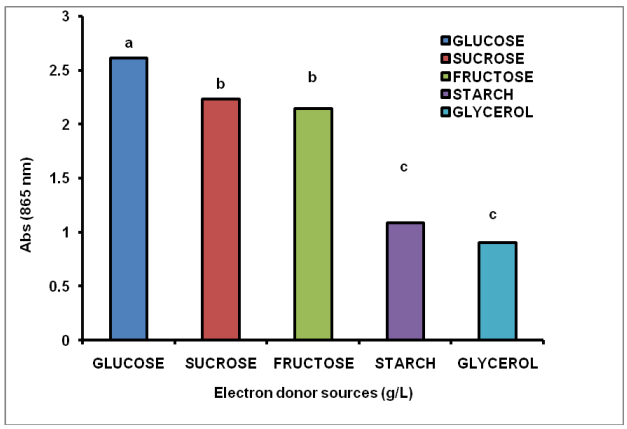

Fig. 2. Effect of various electron donor sources 1% (w/v) on molybdenum reduction by *Enterobacter clocae* after 24 h incubation. Values with different letters over the bars are significantly different ($p<0.05$).

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.

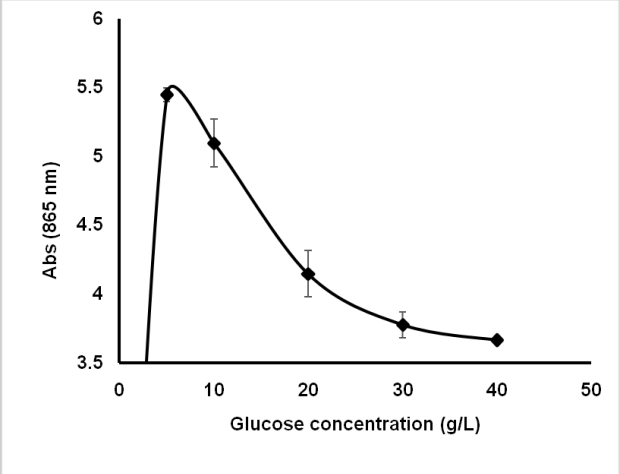

Fig. 3. Effect of different glucose concentrations on molybdenum reduction by *Enterobacter clocae* after 24 h incubation.

Effect of nitrogen source

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, 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.

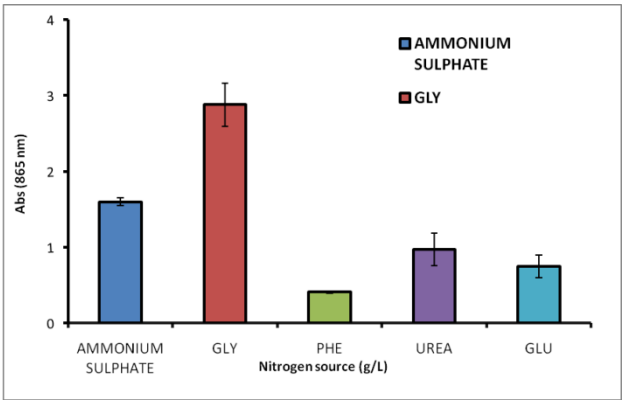

Fig. 4. Effect of various nitrogen sources 0.3% (w/v) on molybdenum reduction by *Enterobacter clocae* after 24 h incubation.

Effect of pH

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 [13].

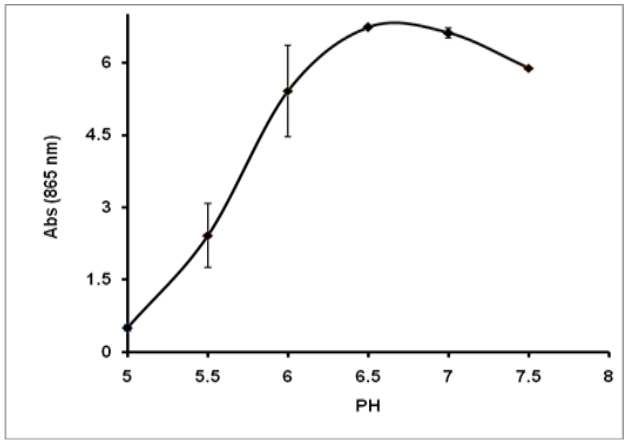

Fig. 5. Effect of different pH on molybdenum reduction by *Enterobacter clocae* after 24 h incubation.

Effect of temperature

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.

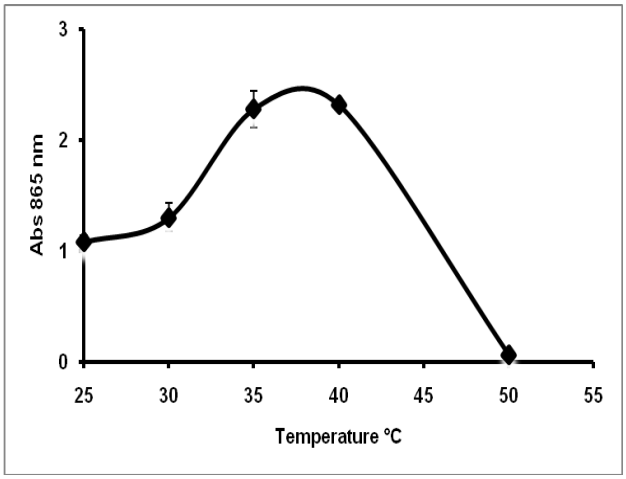

Fig. 6. Effect of different temperature on molybdenum reduction by *Enterobacter clocae* after 24 h incubation.

Effect of phosphate and molybdate concentrations

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 [27]. 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 concentration of molybdenum found in the environment was from an abandoned uranium mine in Colorado with concentration reaching 6,550 mg/Kg [28].

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.

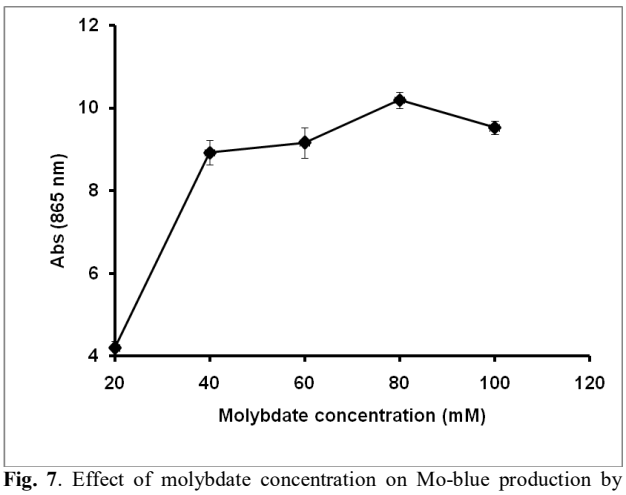

Fig. 7. Effect of molybdate concentration on Mo-blue production by *Enterobacter cloacae* after 24 h incubation at fixed phosphate concentration.

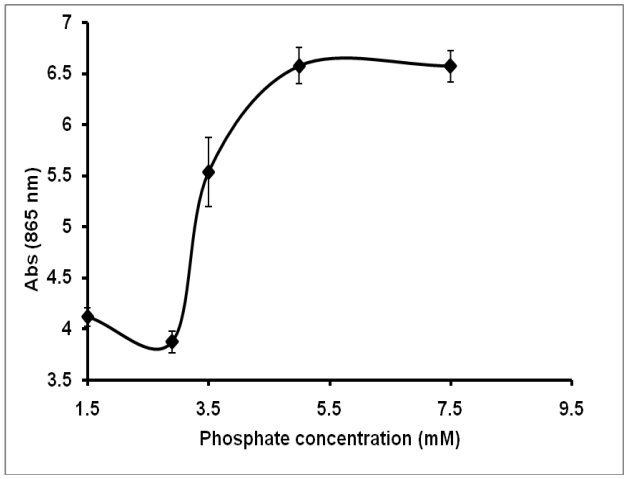

Fig. 8. Effect of phosphate concentrations on molybdenum reduction by *Enterobacter clocae* after 24 h incubation at fixed molybdate concentration.

CONCLUSION

A novel Mo-reducing bacterium has been successfully isolated from agricultural soil. The Gram-negative rod-shape with ability to reduce molybdenum optimally at 5 g/L glucose, pH between 6.5 and 7.0, temperature between 35 and 40 °C, 80 mM sodium molybdate, and phosphate concentration between 5.0 and 7.5 mM. The isolate utilizes glucose and glycine as best carbon and nitrogen sources respectively, and the isolate is tentatively identified as *Enterobacter cloacae* based on phylogenetic analysis of 16S rRNA partial sequencing. This finding will be helpful in future bioremediation using this bacterium.

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