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## Isolation and Characterization of a Metal-reducing *Pseudomonas* sp. strain 135 with Amide-degrading Capability

Yakasai, M.H.<sup>1,2</sup>, Rahman, M.F.<sup>1</sup>, Rahim, M.B.H.A.<sup>1\*</sup>, Khayat, M.E.<sup>1</sup>, Shamaan, N.A.<sup>3</sup> and Shukor, M.Y.<sup>1</sup>

<sup>1</sup>Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia.

<sup>2</sup>Department of Biochemistry, Faculty of Basic Medical Science, Bayero University Kano, P. M. B 3011, Kano state, Nigeria.

<sup>3</sup>Faculty of Medicine and Health Sciences, Islamic Science University of Malaysia, 13th Floor, Tower B, Persiaran MPAJ, Jalan Pandan Utama, Pandan Indah, 55100 Kuala Lumpur, Malaysia.

\*Corresponding author: Mohd. Badrin Hanizam Abd Rahim  
Department of Biochemistry,  
Faculty of Biotechnology and Biomolecular Sciences,  
Universiti Putra Malaysia, 43400 UPM Serdang,  
Selangor, Malaysia.  
Email: [badrin@upm.edu.my](mailto:badrin@upm.edu.my)

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bioremediation  
molybdenum blue

### ABSTRACT

The presence of both heavy metals and organic xenobiotic pollutants in a contaminated site justifies the application of either a multitude of microbial degraders or microorganisms having the capacity to detoxify a number of pollutants at the same time. Molybdenum is an essential heavy metal that is toxic to ruminants at a high level. Ruminants such as cow and goats experience severe hypocuprosis leading to scouring and death at a concentration as low as several parts per million. In this study, a molybdenum-reducing bacterium with amide-degrading capacity has been isolated from contaminated soils. The bacterium, using glucose as the best electron donor reduces molybdenum in the form of sodium molybdate to molybdenum blue. The maximal pH reduction occurs between 6.0 and 6.3, and the bacterium showed an excellent reduction in temperatures between 25 and 40 °C. The reduction was maximal at molybdate concentrations of between 15 and 25 mM. Molybdenum reduction incidentally was inhibited by several toxic heavy metals. Other carbon sources including toxic xenobiotics such as amides were screened for their ability to support molybdate reduction. Of all the amides, only acrylamide can support molybdenum reduction. The other amides; such as acetamide and propionamide can support growth. Analysis using phylogenetic analysis resulted in a tentative identification of the bacterium as *Pseudomonas* sp. strain 135. This bacterium is essential in remediating sites contaminated with molybdenum, especially in agricultural soil co-contaminated with acrylamide, a known soil stabilizer.

### INTRODUCTION

Molybdenum although an essential heavy metal, at an elevated level, it is toxic to ruminants. Numerous industries utilize this element in abundance including the making of alloys, anti-freeze component of an automobile engine and as a lubricant in the form of molybdenum disulphide. The wide application of molybdenum in the industry has resulted in several soils and water pollution cases all around the world such as in the Tokyo Bay, Tyrol in Austria, the Black Sea [1]. Additionally terrestrially, many experts have acknowledged molybdenum as a substantial pollutant in sewage sludge and industrial exhaust fumes [1]. Recently, molybdenum has played a greater role in contributing to the toxicity of organisms through its inhibition of the reproductive processes including spermatogenesis and

embryogenesis in several model organisms [2,3]. Other than heavy metals, manmade chemicals (xenobiotics) of organic pollutants including phenol, amides, pentachlorophenol, sodium dodecyl sulfate (SDS) and hydrocarbons are major global pollutants [4]. Countless tonnes of such chemical substances are created annually with a tremendous amount discovered harming the planet.

The removing of heavy metals and organic contaminants by means of bioremediation is regarded as the most cost-effective strategy in the long run in particular at very low levels, in which various other physical or chemical methods may not be effective [5,6]. Acrylamide is a neurotoxic compound in humans and has been shown in animal models to exhibit carcinogenic and reproductive toxicity [7]. Its usage is as a

monomer for the production of the polyacrylamide polymer. It is from this kind of industry that acrylamide as high as 1 g/L has been reported in the effluent [8]. In Malaysia, a large amount of polyacrylamide is being used annually for the treatment of drinking water. A potential source of acrylamide pollution could be contributed from the use of polyacrylamide in water treatment. The Kuching Water Board in the Sarawak state alone uses nearly one ton of polyacrylamide yearly [9]. Acrylamide pollution can come from the polyacrylamide dispersant used in glyphosate formulation, where degradation to acrylamide in soils has been documented [10].

In the present work, we showed another bacterium with the ability to reduce molybdate to molybdenum blue also showed the capacity to utilize acrylamide as a source of electron donor for molybdenum reduction to molybdenum blue. This simultaneous degradation and reduction make this bacterium falls under the multi-detoxifier category.

## MATERIALS AND METHODS

### Isolation of molybdenum-reducing bacterium

Soil samples from a hydrocarbon contaminated area in a petroleum refinery location were taken (5 cm deep from topsoil). A suspension of soil in tap water was made. An aliquot (0.1 mL) was spread plated onto a low phosphate molybdenum agar media. The composition of the low phosphate media (LPM) at pH 7.0 were as follows: glucose (1%), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.3%), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.05%), yeast extract (0.5%), NaCl (0.5%), Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O (0.242 % or 10 mM) and Na<sub>2</sub>HPO<sub>4</sub> (0.071% or 5 mM) [11]. The plates were then inverted and incubated at room temperature for 24 hours. In the beginning, numerous bacterial colonies formed which some of the, eventually turned into blue colonies. This indicates that these colonies are molybdenum reducers.

The best reducers as judged visually as the most intense blue colony was re-streaked several times to get pure culture. Molybdenum reduction was also carried out in liquid culture medium (at pH 7.0) in a 100 mL LPM in a 250 mL shake flask and cultured at room temperature for 48 h. To study the resultant Mo-blue spectrum, a 1.0 mL of the blue culture medium was taken out, centrifuged at room temperature at 10,000× g for 10 min and scanned between 400 and 900 nm using a Shimadzu 1201UV-spectrophotometer. The baseline correction was low phosphate media.

### 16s rDNA gene sequencing

PCR amplification was carried out on a thermal cycler (Biometra, Gottingen, Germany). Genomic DNA extraction was carried out through the alkaline lysis method. The 16s rDNA forward primer (First Base Sdn Bhd., Malaysia) used was 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer used was 5'-AAGGAGGTGATCCAGCCGCA-3' [12]. The composition, in a final volume of 50 µl of the PCR mixture was as follows: deoxynucleotide triphosphate (200 µM), 1x reaction buffer, primer (0.5 µM) and Taq DNA polymerase (2.5 U) (Promega). PCR operations were carried out as follows: 3 minutes of an initial denaturation at 94 °C; 1 minute each of 25 cycles at 94 °C, 50 °C for 1 minute, 72 °C for 2 minutes, and a final extension at 72 °C for 10 min. A cycle sequencing operation was carried out for the sequencing of the PCR product utilizing the Big Dye terminator kit (Perkin-Elmer Applied Biosystems). The resultant bases were then processed through the GenBank database with the Blast server (<http://www.ncbi.nlm.nih.gov/BLAST/>). The 16s rRNA

ribosomal gene sequence (656 bp) was deposited in the GenBank database under the accession number **EF121819**.

### Identification of bacterium

Identification of the bacterium was carried out through a molecular phylogenetic analysis. First, multiple alignments of the 16S rRNA gene sequences from closely matched retrieved sequences from GenBank was carried out (PHYLIP output format) using the Clustal\_Omega program [13]. The phylogenetic tree was developed by using the PHYLIP suits of the program (Felsenstein, <http://evolution.genetics.washington.edu/phylip.html> Department of Genetics, University of Washington, Seattle, WA., v. 3.573.).

A distance-based method was utilized to calculate the phylogenetic relationship. The evolutionary distance matrices from the neighbour-joining/UPGMA method was calculated using the DNADIST algorithm program. The program produces an output file of the distance matrix. The output file was then analysed using the Jukes and Cantor model of nucleotide substitution [14]. The neighbour-joining method of Saitou and Nei was employed to infer the phylogenetic tree [15]. A 1000 bootstraps repetition for each algorithm were carried out to checked for confidence levels of the individual branches of the tree. This was executed using the SEQBOOT program [16]. A majority rule consensus trees (50%) from a series of trees for the topologies found were constructed in the form of Newick standard format using the CONSENSE program. Finally, the TreeView program was utilized to view the tree [17].

### Preparation of resting cells for molybdenum reduction characterization

Characterization works on molybdenum reduction to Mo-blue such as the effects of pH, temperature, phosphate and molybdate concentrations were carried out statically using resting cells in a microplate or microtiter format as previously developed [18]. Cells from a 1 L overnight culture grown in High Phosphate media (HPM) at room temperature on an orbital shaker (150 rpm) with the only difference between the LPM and HPM was the phosphate concentration which was fixed at 100 mM for the HPM. Cells were harvested by centrifugation at 15,000 × g for 10 minutes and the pellet was washed several times to remove residual phosphate and resuspended in 20 mls of low phosphate media (LPM) minus glucose to an absorbance at 600 nm of approximately 1.00.

In the low phosphate media, a concentration of 5 mM phosphate was maximal for all of the Mo-reducing bacteria isolated so far and hence this concentration was used in this work. Higher concentrations were found to be strongly inhibitory to molybdate reduction [11,19–33]. Then 180 µL was sterically pipetted into each well of a sterile microplate. 20 µL of sterile glucose from a stock solution was then added to each well to initiate Mo-blue production. A sterile sealing tape that allows gas exchange (Corning® microplate) was used for sealing the tape. The microplate was incubated at room temperature. At defined times absorbance at 750 nm was read in a BioRad (Richmond, CA) Microtiter Plate reader (Model No. 680). The production of Mo-blue from the media in a microplate format was measured using the specific extinction coefficient of 11.69 mM<sup>-1</sup>.cm<sup>-1</sup> at 750 nm as the maximum filter wavelength available for the microplate unit was 750 nm [34].

### Screening of molybdenum reduction using xenobiotics as source of electron donor

The ability of xenobiotics such as phenol, acrylamide, nicotinamide, acetamide, iodoacetamide, propionamide, Sodium Dodecyl Sulfate (SDS) and diesel to support molybdenum reduction as electron donors was tested using the microplate format above by replacing glucose from the low phosphate medium with these xenobiotics at the final concentration of 500 mg/L taking into account the general toxicity of these xenobiotics. Diesel was initially added to the final concentration of 0.5 g/L in 10 mL media and sonicated for 5 minutes. Then 200  $\mu$ L of the media was added to the microplate wells.

#### Effect of heavy metals on molybdenum reduction

Seven heavy metals namely lead (ii), arsenic (v), copper (ii), mercury (ii), silver (i), chromium (vi) and cadmium (ii) were prepared either from commercial salts or standard solutions of Atomic Absorption Spectrometry (MERCK). The bacterium was incubated with heavy metals in the microplate format at various concentrations. The plate was incubated for 24 hours at 30 °C. The amount of Mo-blue production was measured at 750 nm as before.

#### Screening for bacterial growth on xenobiotics independent of molybdenum reduction

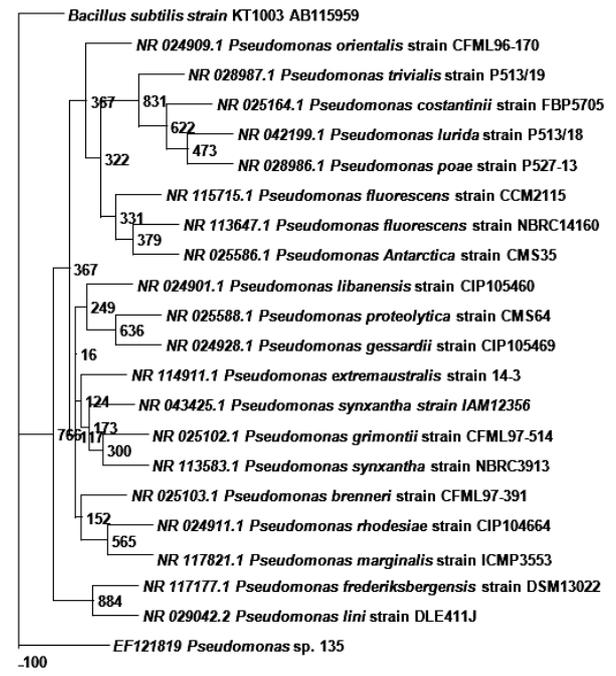
The ability of xenobiotics such as phenol, acrylamide, nicotinamide, acetamide, iodoacetamide, propionamide, Sodium Dodecyl Sulfate (SDS) and diesel to support bacterial growth independent of molybdenum reduction was tested using the microplate format above using the following media with these xenobiotics at the final concentration of 500 mg/L. The ingredients of the growth media with the high concentration of molybdenum omitted, as it might have a detrimental effect on growth on xenobiotics, were as follows:  $(\text{NH}_4)_2\text{SO}_4$  (0.3%),  $\text{NaNO}_3$  (0.2%),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.05%), yeast extract (0.01%),  $\text{NaCl}$  (0.5%),  $\text{Na}_2\text{HPO}_4$  (0.705% or 50 mM) and 1 mL of trace elements solution [35] with composition (mg/L) as follows:  $\text{CaCl}_2$  (40),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (40),  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  (40),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (20),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (5),  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (5),  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (5). The media was adjusted to pH 7.0. The increase of bacterial growth after an incubation period of 3 days at room temperature was measured at 600 nm using the microplate reader (Bio-Rad 680).

#### Statistical analysis

Values are means  $\pm$  SE. Data analyses were carried out using Graphpad Prism version 3.0 and Graphpad InStat version 3.05 available from www.graphpad.com. A Student's t-test or one-way analysis of variance with post hoc analysis by Tukey's test was employed for comparison between groups.  $P < 0.05$  was considered statistically significant.

## RESULTS AND DISCUSSIONS

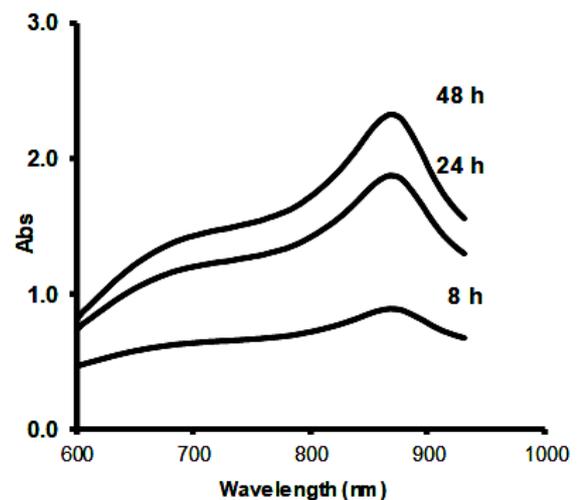
Strain 135 was independently not associated to other clades harbouring several *Pseudomonas* species such as *Pseudomonas* spp., *Pseudomonas grimontii*, *Pseudomonas rhodesiae*, *Pseudomonas Antarctica*, *Pseudomonas poae*, *Pseudomonas putida*, *Pseudomonas veronii* and *Pseudomonas marginalis* demonstrating that strain 135 does not belong to any specific *Pseudomonas* species (Fig. 1). For now, isolate 135 is assigned tentatively as *Pseudomonas* sp. strain Dr.Y135. This is not the first *Pseudomonas* strains with the ability to reduce molybdenum as two Mo-reducing bacteria from this genus, which is *Pseudomonas* sp. strain DRY2 [26] and *Pseudomonas* sp. strain DRY1, an Antarctic bacterium [30] are both molybdenum reducers.



**Fig 1.** A neighbour-joining method phylogram, which shows the genetic relationship between strain 135 and 20 closest bacterial sequence. Species names are preceded by the accession numbers of their 16S rRNA sequences. The bootstrap values are indicated by the numbers at branch points which are based on 1000 re-samplings. The scale bar signifies 100 nucleotide substitutions. The outgroup is *B. subtilis*.

#### Molybdenum absorbance spectrum

When the Mo-blue produced by *Pseudomonas* sp. strain 135 was scanned over the entire visible and near infra-red region, a unique characteristics spectrum with a shoulder at approximately 700 nm and a maximum peak with a median at 865 nm near the infra-red region of between 860 and 870 nm was observed which was conserved in the early and late culturing process (Fig. 2). We have discussed previously that this unique spectrum indicates that it is a reduced phosphomolybdate [22] and was suggested by Campbell et al. [19] previously. It is not easy to indicate the true identity of the reduced phosphomolybdate as there are numerous species of this compound [36].



**Fig. 2.** Scanning absorption spectrum of Mo-blue from *Pseudomonas* sp. strain 135 at different time intervals.

### Effect of pH and temperature on molybdate reduction

Based on ANOVA analysis it was found that the optimum pH for molybdate reduction was between 6.0 and 6.3 with a dramatic loss of reducing activity at pH lower than 5 (Fig. 3). The effect of temperature observed between 20 and 60 °C (Fig. 4) shows that temperatures ranging from 25 to 40 °C gave maximal Mo-blue production as measured with no significant difference ( $p>0.05$ ) among the values measured. It was also discovered that temperatures exceeding 40 °C cause a strong inhibition of the Mo-blue production from this bacterium.

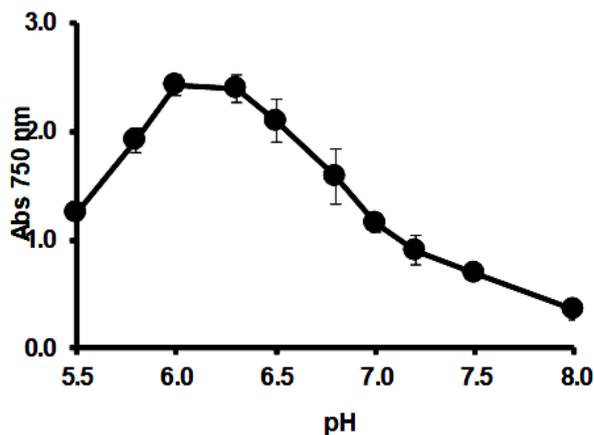


Fig. 3. Effect of pH on molybdenum reduction by *Pseudomonas* sp. strain 135 studied using a resting cells preparation and incubated for 72 h. Error bars represent mean  $\pm$  standard deviation ( $n=3$ ).

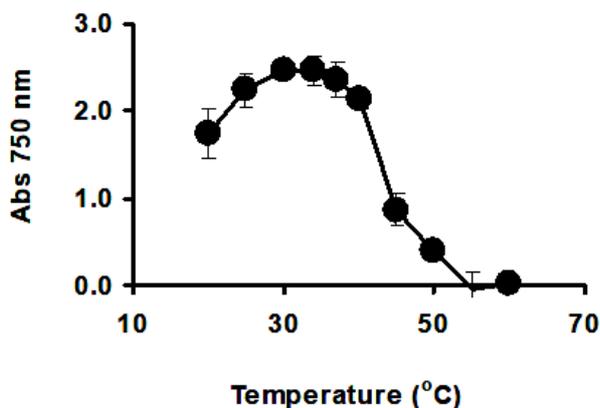


Fig. 4. Effect of temperature on molybdenum reduction by *Pseudomonas* sp. strain 135 studied using a resting cells preparation and incubated for 72 h. Error bars represent mean  $\pm$  standard deviation ( $n=3$ ).

Temperature and pH affect molybdenum reduction; an enzyme-mediated process. The optimum conditions obtained in this work would be an advantage for bioremediation to be conducted in a tropical country like Malaysia with average yearly temperature overlapping the maximum range obtained in this study [21]. Therefore, *Pseudomonas* sp. strain 135 could be a candidate for soil bioremediation of molybdenum locally and in other tropical countries. The majority of the molybdenum reducers exhibit similar maximal temperature range for supporting molybdenum reduction and with the only psychrotolerant reducer isolated from Antarctica showing a range of between 15 and 20 °C [30].

The maximal pH range exhibited by *Pseudomonas* sp. strain 135 encompasses the acidic pH requirement observed in many molybdenum reducers and is hypothesized to play an

important role as acidic conditions stabilize the phosphomolybdate and assist in its formation before it is being reduced to Mo-blue [22].

### Effect of electron donor on molybdate reduction

Carbon sources that are easily assimilable like glucose are important products of the reducing equivalents NADH and NADPH that are direct electron donors to phosphomolybdate, leading to its reduction to Mo-blue [37]. Hence, it was not surprising that glucose was the best electron donor for supporting molybdate reduction as observed in many of the molybdenum reducers [38,39]. This is followed in descending order of efficiency by galactose and fructose (Fig. 5). Other carbon sources did not support molybdenum reduction.

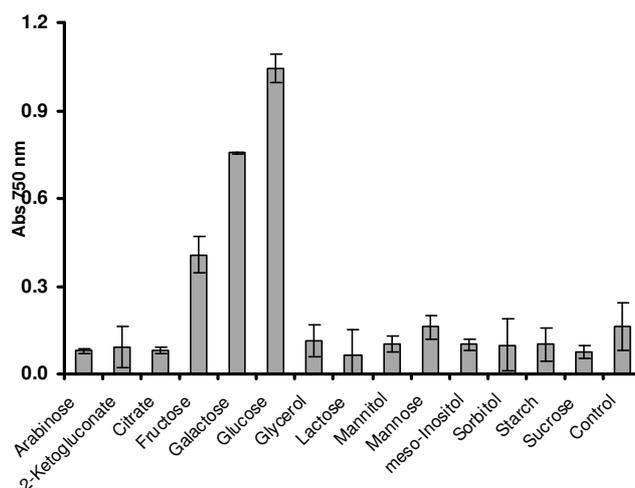


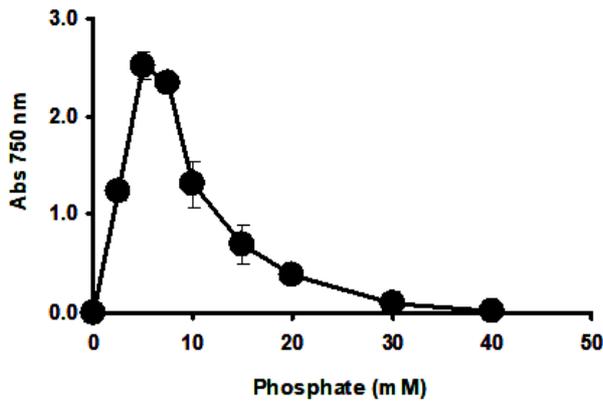
Fig. 5. Effect of different electron donor sources (1% w/v) on molybdenum reduction. *Pseudomonas* sp. strain 135 was grown in low phosphate media containing 10 mM molybdate and various electron donors for 72 h. Error bars represent mean  $\pm$  standard deviation ( $n = 3$ ).

### Molybdenum reduction in the presence of high concentration of phosphate and molybdate ions

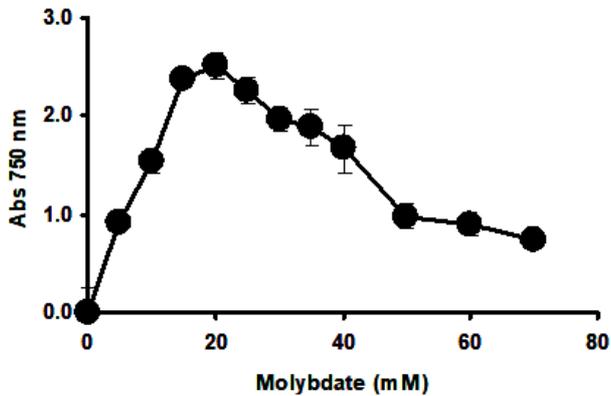
Phosphate is known to inhibit molybdenum reduction in many Mo-reducing bacteria with the inhibitory concentration varying from 2.9 to 7.5 mM [11,21,23,24,26–28,30,32,37,39]. In this study, it was found that a similar situation was observed with phosphate inhibiting reduction at between 5.0 and 7.5 mM. Similarly, the very high concentration of phosphate inhibits reduction (Fig. 6). The reason for this inhibition is because phosphate is known to disrupt the stability of the phosphomolybdate ions [40–42]. Studies on the effect of molybdenum concentration on molybdenum reduction are important as it was reported that in a molybdenum and uranium mine, the soils surrounding the area in Colorado [43] showed the extremely high concentration of molybdenum reaching about 6,500 mg/Kg (approximately 68 mM).

Two previously isolated molybdenum reducers can reduce the very high concentration of molybdenum at 80 mM as seen in *E. coli* K12 [19] and *Klebsiella oxytoca* strain hkeem [28]. Hence, high molybdenum resistant bacterium with the ability to reduce molybdenum at this concentration is highly sought. The results showed that the newly isolated bacterium could reduce molybdenum as high as 60 mM but the reduction activity was severely affected. The maximal reduction range was between 15 and 25 mM (Fig. 7). This is within the range of many molybdenum-reducing bacteria such as

*Pseudomonas* sp strain Dr.Y2 at 15 mM [26] and *S. marcescens* strain Dr.Y6 at 25 mM [21].



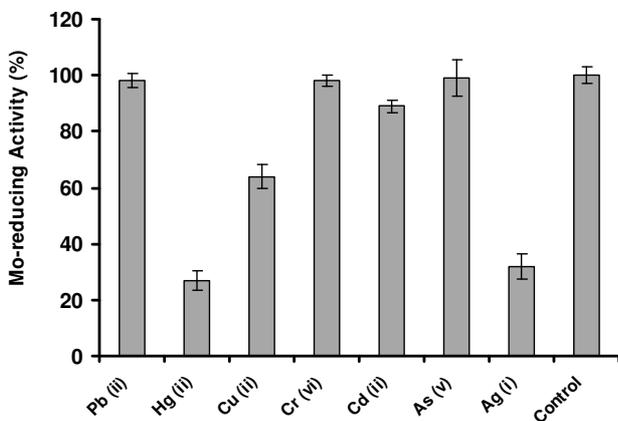
**Fig. 6.** Inhibitory effect of phosphate on molybdenum reduction by *Pseudomonas* sp. strain 135 after 72 h of incubation. Error bars represent mean ± standard deviation (n = 3).



**Fig. 7.** The effect of molybdate concentration on molybdenum reduction by *Pseudomonas* sp. strain 135 after 72 h of incubation. Error bars represent mean ± standard deviation (n = 3).

**Effect of heavy metals**

The inhibitory actions of toxic metal ions on bioremediation, in general, has not been given the exposure it deserves with numerous biodegradation and even bioreduction activity is inhibited by non-target heavy metals. This study aims to assess the inhibitory effect of heavy metals on molybdenum reduction. The heavy metals tested were Hg (ii), Ag (i), Cu (ii), Cd (ii) at 2 ppm and the resultant inhibition by these heavy metals were 73.2, 68.2, 36.0 and 11.0%, respectively (Fig. 8).



**Fig. 8.** The effect of metal ions on molybdenum blue production by *Pseudomonas* sp. strain 135 after 72 h of incubation. Error bars represent mean ± standard deviation (n = 3).

These toxic heavy metals are known to inhibit molybdenum reduction as described previously with mercury founds as a physiological inhibitor to molybdate reduction [44]. A list (Table 1) of the type of heavy metals that inhibit molybdenum reduction in various Mo-reducing bacteria is shown. The sulfhydryl group of enzymes are normally the site of inhibition by heavy metals such as mercury, cadmium, silver and copper [45].

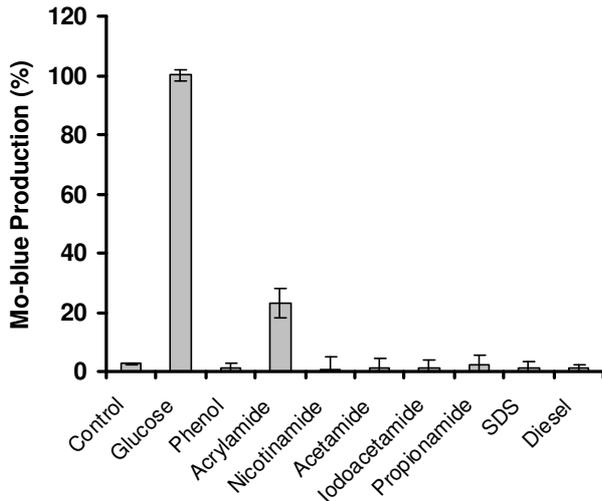
**Table 1.** Inhibition of Mo-reducing bacteria by heavy metals.

Bacteria	Heavy Metals that inhibit reduction	Author
<i>Bacillus pumilus</i> strain lbna	As <sup>3+</sup> , Pb <sup>2+</sup> , Zn <sup>2+</sup> , Cd <sup>2+</sup> , Cr <sup>6+</sup> , Hg <sup>2+</sup> , Cu <sup>2+</sup>	[29]
<i>Bacillus</i> sp. strain A.rzi	Cd <sup>2+</sup> , Cr <sup>6+</sup> , Cu <sup>2+</sup> , Ag <sup>+</sup> , Pb <sup>2+</sup> , Hg <sup>2+</sup> , Co <sup>2+</sup> , Zn <sup>2+</sup>	[32]
<i>Serratia</i> sp. strain Dr.Y8	Cr, Cu, Ag, Hg	[24]
<i>S. marcescens</i> strain Dr.Y9	Cr <sup>6+</sup> , Cu <sup>2+</sup> , Ag <sup>+</sup> , Hg <sup>2+</sup>	[11]
<i>Serratia</i> sp. strain Dr.Y5	n.a.	[25]
<i>Pseudomonas</i> sp. strain DRY2	Cr <sup>6+</sup> , Cu <sup>2+</sup> , Pb <sup>2+</sup> , Hg <sup>2+</sup>	[26]
<i>Pseudomonas</i> sp. strain DRY1	Cd <sup>2+</sup> , Cr <sup>6+</sup> , Cu <sup>2+</sup> , Ag <sup>+</sup> , Pb <sup>2+</sup> , Hg <sup>2+</sup>	[30]
<i>Enterobacter</i> sp. strain Dr.Y13	Cr <sup>6+</sup> , Cd <sup>2+</sup> , Cu <sup>2+</sup> , Ag <sup>+</sup> , Hg <sup>2+</sup>	[23]
<i>Acinetobacter calcoaceticus</i> strain Dr.Y12	Cd <sup>2+</sup> , Cr <sup>6+</sup> , Cu <sup>2+</sup> , Pb <sup>2+</sup> , Hg <sup>2+</sup>	[27]
<i>Serratia marcescens</i> strain DRY6	Cr <sup>6+</sup> , Cu <sup>2+</sup> , Hg <sup>2+</sup> *	[21]
<i>Enterobacter cloacae</i> strain 48	Cr <sup>6+</sup> , Cu <sup>2+</sup>	[20]
<i>Escherichia coli</i> K12	Cr <sup>6+</sup>	[19]
<i>Klebsiella oxytoca</i> strain hkeem	Cu <sup>2+</sup> , Ag <sup>+</sup> , Hg <sup>2+</sup>	[28]

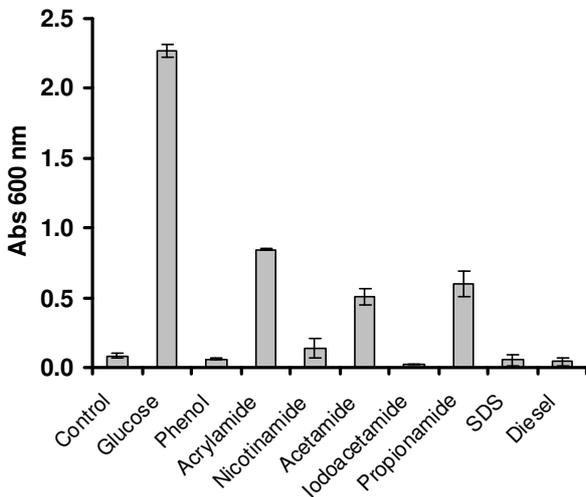
**Xenobiotics as either electron donors for molybdenum reduction or supporting growth**

As more and more studies are targeting on the isolation of multi-reducers. The ability of various xenobiotics to support molybdenum reduction was explored. Of all the xenobiotics tested, only acrylamide was shown to support molybdenum reduction at a lower efficiency than glucose while other xenobiotics tested could not (Fig. 9). The use of acrylamide as an electron donor source is important for simultaneous removal of the two pollutants without the addition of further carbon sources which can be costly.

On the other hand, the bacterium was able to grow on acrylamide, acetamide and propionamide (Fig. 10), which means that these pollutants can also be degraded and removed independently of molybdenum reduction. The search for bacteria with multiple degrading capacities will be very useful in bioremediation works and very few bacteria that could simultaneously detoxify heavy metals and degrade xenobiotics. An example is in chromate reduction where xenobiotics such as phenol and pyridine can act as electron donors for chromate reduction [4,46].



**Fig. 9.** Mo-blue production by xenobiotics at 10 mM as an electron donor in low phosphate media. Glucose was a positive control. The incubation period was 72 h. Error bars represent mean  $\pm$  standard deviation (n = 3).



**Fig. 10.** The growth of *Pseudomonas* sp. strain 135 on various xenobiotics. Glucose was a positive control, and molybdenum was not included in the media. Incubation was 72 h. Error bars represent mean  $\pm$  standard deviation (n = 3).

## CONCLUSION

In this study, we report on the isolation of a Mo-reducing bacterium that can use acrylamide as a source of electron donor. The bacterium reduces molybdate to Mo-blue maximally at sub neutral pH and a broad temperature range of between 25 and 40 °C. The most easily assimilable carbon source; glucose was the best electron donor while acrylamide, a toxic xenobiotic can also be an electron donor but at a much-reduced efficiency. Another important result was the ability of the bacterium to grow on acrylamide, acetamide and propionamide as a carbon source. Currently, work is underway to optimize reduction using response surface method (RSM) and to purify the molybdenum-reducing enzyme from this bacterium.

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