



## Molybdate Reduction to Molybdenum Blue and Growth on Polyethylene Glycol by *Bacillus* sp. strain Neni-8

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### ABSTRACT

The accumulation of heavy metals and xenobiotic compounds in soil and aquatic bodies is caused by inappropriate waste disposal, industrial and mining operations, and excessive use of agricultural pesticides. Bioremediation is a more cost-effective way of removing these pollutants than other approaches. A new molybdenum-reducing bacterium with the ability to grow on a variety of polyethylene glycol (PEG)s has been discovered. Based on biochemical test, the bacterium was partially identified as *Bacillus* sp. strain Neni-8. Mo-blue production required an optimal pH of between 6.3 and 6.5, and between 30 and 37 °C. The carbon source, D-glucose best supported molybdenum reduction. A narrow requirement for phosphate of between 2.5 and 7.5 mM for molybdenum reduction was seen. Sodium molybdate as a substrate for reduction showed maximal reduction between 20 and 30 mM. The molybdenum blue absorption spectrum indicates that its identity was possibly a reduced phosphomolybdate. Several heavy metals such as silver, mercury, copper and chromium inhibited molybdenum reduction by 67.6, 48.7, 36.8 and 17.4 %, respectively. Bacterial growth modelled using the modified Gompertz model with PEG 600 as the best carbon source predicted a maximum growth rate of 15.4 Ln CFU/ml, a maximum specific growth rate of 0.198 h<sup>-1</sup> and a lag period of 10.1 h. The novel characteristics of this bacterium are very useful in future bioremediation works.

### INTRODUCTION

The presence of heavy metals and xenobiotics chemicals such as hydrocarbons, dyes and polymers in the environment is caused by the improper disposals and managements of industrial, agricultural and mining wastes. The removal of these contaminants through bioremediation can be much cheaper than other methods, including physical or chemical methods in the long run, and is also environmentally friendly. Polyethylene glycols are synthetic polymers, and are used in many industries such as cosmetics, lubricants, pharmaceuticals, and antifreeze for automobile radiators and in the production of non-ionic surfactants. PEG is nephrotoxic. Wounded rabbit exposed

topically to polyethylene glycol-based antimicrobial cream model showed evidence of nephrotoxicity with symptoms of failure. Several of the animals tested died within one week of therapy [1]. About millions of tons of PEGs are manufactured globally. Effluents contaminated with PEGs usually reach conventional sewage treatment systems making them a significant pollutant [2].

PEGs are water-soluble polymers but the difference is in their molecular weights. Since their introduction more than thirty years ago, the fate of these polymers is of concern, and several biodegradability studies showed some promise in their removal from the environment. Biodegradation of PEG was first

documented in 1965 [2] and further isolations of PEG-degrading microorganisms have been reported [2].

Industrial and mining activities are two of the most frequently activities associated with molybdenum contamination of the environment. In Armenia, waste effluents from the Alaverdi copper molybdenum mine have caused the contamination of land with an area estimated as 300 square kilometres [3]. Mining activities in southern Colorado (USA) led to the presence of molybdenum in surrounding areas to a concentration as high as 2,000 mg/L in the soils. The Water Quality Control Commission stated that several miles of the Red River are considered dead due to pollution [4]. In an abandoned uranium mine in North Cave Hills South Dakota, (USA), a soil sample taken from the site in 2004 showed molybdenum concentration as high as 6,550 mg/Kg [5].

In Jordan, extensive oilshale exploration coupled with intensive agriculture have caused molybdenum pollution with soils exhibiting molybdenum level as high as 11.7 mg/kg [6]. Molybdenum is mined in Malaysia as a by-product of copper mining. Ruptures of pipes carrying metal wastes have been reported to cause serious environmental issues [7]. In Batu Hijau, Sumbawa, Indonesia, copper and gold mining activity from a copper-gold-molybdenum porphyry deposit is slowly polluting the surrounding coastal areas with heavy metals from mine tailings dumped into the sea. ow reduced fish populations and water pollution between 2006 and 2010; the mine dumps over several million tonnes of tailings into the ocean every year [8,9]. Industrial activities have led to reports of molybdenum pollution the waters of the Tokyo Bay and the Black Sea [10], and agricultural soils in Tyrol, Austria [11].

Of all of the animals, ruminants are the most sensitive to molybdenum. Exposure to molybdenum at levels of between 5 to 10 ppm resulted in hypocuprosis or copper deficiency. In the rumen of these animals, molybdenum is converted to thiomolybdate compounds, which are good chelators of copper. As a result the animals experience copper deficiency, which can lead to scouring and even deaths [12]. Evidences have begun to reveal the toxicity of molybdenum to spermatogenesis. Observations from several organisms such as drosophila [13], rats [14] and the Japanese eel (*Anguilla japonica*) [15] have shown that molybdenum affected spermatogenesis at concentrations of between 50 to 150 mg/Kg when fed in the diet. Studies in drosophila showed that molybdenum exhibits the most distinct genetic action at the initial phases of spermatogenesis [13].

Molybdenum can be removed from solution through chemical, physical and biological methods. Of all these methods, biological removal via bacterial reduction is gaining more attention due to economic factors. Bacterial reduction the insoluble molybdenum disulphide [16] by sulphate reducing bacteria, and to the colloidal molybdenum blue [17] are candidates for molybdenum bioremediation. Of the two, the latter is preferred as the bacterial conversion of molybdenum to Mo-blue can be carried out under facultative anaerobic conditions instead of a complete anaerobic conditions in the former [18]. Mo-reducing bacterial candidates suitable for bioremediation have been reported such as *E. coli* K12 [19], *Acinetobacter* [20], and bacteria from the genera of *Enterobacter* [18,21], *Klebsiella* [22–24], *Bacillus* [25,26], *Pseudomonas* [27,28], and *Serratia* [29–32]. A Mo-reducing bacterium isolated from polluted Indonesian soil shows the ability to use various PEGs as carbon sources for growth. The

novel characteristics of the bacterium are very useful in future bioremediation works.

## MATERIALS AND METHODS

### Growth, isolation and characterization of Mo-reducing bacterium

Samples were collected about 5 cm deep from the topsoil, taken from an industrial site in Bukittingi, West Sumatra, Indonesia, in January 2009 by the late Dr Neni Gusmanizar. One gram of soil sample was suspended in 10 mL of sterile tap water. An aliquot of 0.1 mL of the soil suspension was then spread onto an LPM agar or low phosphate media (pH 7.0) and incubated for 48 h at room temperature. The LPM was composed (w/v) as follows: glucose (1%), NaCl (0.5%), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.05%), yeast extract (0.5%), Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O (0.242 % or 10 mM), (NH<sub>4</sub>)<sub>2</sub>.SO<sub>4</sub> (0.3%), agar (1.5%), and Na<sub>2</sub>HPO<sub>4</sub> (0.071% or 5 mM) [32]. Molybdenum reduction was indicated by the presence of blue colonies.

The colony with the most intense blue was restreaked on the LPM agar to obtain pure culture. A liquid culture was also utilized to monitor Mo-blue production. The blue supernatant was scanned from 400 to 900 nm (UV-spectrophotometer, Shimadzu 1201) with uninoculated media as baseline blank. Biochemical and phenotypical identification methods according to the Bergey's Manual of Determinative Bacteriology [33] and the ABIS online system [34] were carried out in the process to identify the bacterium.

### Bacterial resting cells preparation

As previously done, the study for optimum conditions necessary for molybdenum reduction in this bacteria used resting cells in a microplate (microtiter) format [35]. To prepare resting cells without the presence of blue product, the LPM medium above was modified by excluding sodium molybdate and increasing the phosphate concentration to 100 mM. Overnight growth from a single colony inoculation was carried out at 120 rpm on an orbital shaker (Yihder, Taiwan). Cells were centrifuged at 15,000 x g for 10 min. Pelleted cells were rinsed twice briefly with deionized water and resuspended in 20 mL of LPM with glucose omitted.

During the characterization process, appropriate changes to the LPM were made to address differences in phosphate, molybdate, and pH conditions. Cells were first thoroughly homogenized and 180 µL was transferred into the wells of a sterile microplate. Then 20 µL of sterile glucose or other carbon sources were added from a stock solution to the final concentration of 1.0 % (w/v). The final volume was 200 µL. Addition of the carbon sources started Mo-blue production. Incubation was carried out at room temperature. Growth was measured at 600 nm while Mo-blue reduction was monitored at 750 nm (BioRad (Richmond, CA) Microtiter Plate reader (Model No. 680). The readings at 750 nm must first be subtracted from readings at 600 nm to measure Mo-blue. The specific extinction coefficient of 11.69 mM<sup>-1</sup>.cm<sup>-1</sup> at 750 nm was utilized to quantify Mo-blue production. This wavelength is the maximum filter available for the microplate unit [35]. The effect of several heavy metals was studied utilizing Atomic Absorption Spectrometry calibration standard solutions from MERCK.

### PEGs as carbon sources for growth

Various polyethylene glycols compounds from PEG 200 to PEG 20,000 (Sigma Aldrich, St. Louis, U.S.A.) were tested for

their ability to act as electron donors for molybdenum reduction. Since none of the compound can support reduction based on preliminary results, the compounds were tested for their capacity to support growth of this bacterium. Briefly, glucose was replaced from the microplate above and replaced with various PEGs dissolved in HPM (minus sodium molybdate) at the final concentration of 500 mg/L. A of 50  $\mu$ L of bacterial suspension was utilized. The final mixture had a total volume of 250  $\mu$ L. The growth media was adjusted to pH 7.0. The microplate was sealed as before. The increase of bacterial growth after an incubation period of 10 days at room temperature was monitored at 600 nm. Monitoring of the best PEG as a carbon source was carried out utilizing aerobic growth on HPM at room temperature, and shaken at 120 rpm on an orbital shaker. Growth was modelled according to the modified Gompertz model [36].

#### PEG degradation assay

At a concentration of 10 mg/L, stock solutions of different PEGs were produced. The total volume was 2 mL and was made up of suitably diluted stock solutions of different PEGs ranging from 1 to 7.5 mM. Before adding the test reagents, samples from the resting cell preparation were diluted appropriately. The culture was incubated for 10 d statically at room temperature. PEG was quantified using a reagent consisting of a 200  $\mu$ L solution of 5% (w/v)  $\text{BaCl}_2 \cdot \text{H}_2\text{O}$  in 1 M HCl mixed with 200  $\mu$ L of a solution of 1.27g iodine dissolved in 100 ml of 2% (w/v) potassium iodide. After mixing, the reaction mixture was incubated at room temperature for 10 min against a reagent blank made in the same way but with water instead of sample. A spectrophotometer was used to read the mixture at 535 nm. The intensity of brownish orange hue formed by the complex solution was used to measure the PEG content. This technique is sensitive, straightforward, and universally applicable to all PEG derivatives [37].

#### Statistical analysis

ANOVA with Tukey's post hoc analysis was carried out using Graphpad Prism version 5.0.

### RESULTS AND DISCUSSIONS

The reduction of molybdate to molybdenum blue by bacteria was first described more than one hundred years ago in 1896 by Capaldi, and Proskauer [17]. Further isolation of Mo-reducing bacteria in the last century were reported in 1939 [38], in 1948 [39], in 1962 [40], in 1972 [41], in 1985 [19], and in 1993 [18]. Ghani et al. [18] quickly recognize the potential application of this phenomenon for the bioremediation of molybdenum. Since then, numerous Mo-reducing bacteria have been isolated [42]. The ability of the newly isolated Mo-reducing bacterium to grow on other xenobiotics is indeed a highly sought-after property.

#### Mo-reducing bacterium identification

Gram staining showed that the bacterium was Gram-positive, rod-shaped and motile. The bacterial identification was suggested by the ABIS online program (Table 1) with accuracy at 94% and homology (91%), as either *Bacillus subtilis* or *Bacillus atropheus*. Hence, identification up to the species level was not feasible. The bacterium was identified as *Bacillus* sp. strain Neni-8 in honor of the late Dr. Neni Gusmanizar who did the soil sampling. The 16s rRNA gene from this bacterium is now being amplified and sequenced in order to do phylogenetic analysis and better identify the bacterium.

**Table 1.** Biochemical tests for *Bacillus* sp. strain Neni-8.

Gram positive staining	+	Acid production from:	
Motility	+	N-Acetyl-D-Glucosamine	d
Growth at 45 °C	+	L-Arabinose	+
Growth at 65 °C	-	Cellobiose	+
Growth at pH 5.7	+	Fructose	+
Growth on 7% NaCl media	+	D-Glucose	+
Anaerobic growth	-	Glycerol	+
Casein hydrolysis	+	Glycogen	+
Esculin hydrolysis	+	meso-Inositol	+
Gelatin hydrolysis	+	Lactose	d
Starch hydrolysis	+	Mannitol	+
Tyrosine degradation	-	D-Mannose	+
Beta-galactosidase (ONPG)	+	Maltose	+
Catalase	+	Melezitose	-
Oxidase	d	Melibiose	d
Urease	-	Raffinose	+
Arginine dehydrolase (ADH)	-	Rhamnose	-
Lysine decarboxylase (LDC)	-	Ribose	+
Ornithine decarboxylase (ODC)	-	Salicin	+
Indole production	-	Sorbitol	+
Citrate utilization	+	Sucrose	+
Egg-yolk reaction	-	Starch	+
Nitrates reduction	+	Trehalose	+
Voges-Proskauer test (VP)	+	D-Xylose	+

Note: + positive result, - negative result, d indeterminate result

Several Mo-reducing bacteria belonging to this genus have been reported namely *Bacillus pumilus* strain lbna [25], *Bacillus* sp. strain khayat [43], *Bacillus* sp. strain A.rzi [26], *Bacillus* sp. strain Zeid 14 [44], *Bacillus tequilensis* strain Pharon2 (MK078034) from Egypt and three *Bacillus* spp. Isolated from similar sites previously which are *Bacillus* sp. strain Neni-12 and [45]; *Bacillus* sp. strain Neni-10 [46,47] and *Bacillus amyloliquefaciens* strain Neni-9 [48], have been isolated.

The use of resting cells in molybdenum reduction research is advantageous since every Mo-reducing bacterium isolate discovered so far reduces molybdenum best under static or low oxygen tension circumstances, and it was initially used in *Enterobacter cloacae* strain 48 [18]. The use of a microtiter plate enables for a high-throughput characterization of the reduction process [35,49]. For example, in selenate reduction, researchers have used resting cells under static circumstances to describe metal reduction and xenobiotics degradation in bacteria [50] and SDS biodegradation [51].

#### Molybdenum absorbance spectrum

The bacterium exhibited a molybdenum blue spectrum with a maximum peak at 865 nm and a shoulder at 700 nm. As Mo-blue production increases, a conservation of this unique profile was observed (Fig. 1). The Mo-blue spectrum appeared identical to the spectrum of molybdenum blue from the phosphate determination method, which exhibits a peak maximum near 890 nm and a characteristics shoulder at 700 nm.

The Mo-blue produced from the phosphate determination method is a reduced phosphomolybdate [52,53]. We discovered that the Mo-blue spectra from almost all of the Mo-reducing bacteria isolated to date are similar to the Mo-blue from the phosphate determination method [24,54]. Previously, we have suggested that this similarity indicates that during bacterial reduction of molybdate to Mo-blue, a phosphomolybdate intermediate exists [54]. The necessity for an intermediate

species in molybdate reduction can also be observed in relevant works on biological chromate reduction.

In no less than two bacteria such as *Pseudomonas ambigua* [55] and *Shewanella putrefaciens* (now known as *S. oneidensis*) [56], the reduction of  $\text{Cr}^{6+}$  to  $\text{Cr}^{3+}$  goes through an unstable intermediate species,  $\text{Cr}^{5+}$  [57]. This phenomenon seen is probably due to the close similarity in chemistry between molybdate and chromate ions [58]. Even though nuclear magnetic resonance and electron spin resonance analyses are needed to correctly identify the lacunary species of phosphomolybdate involved, spectroscopic technique would be generally enough to distinguish the broad category existing amongst various heteropolymolybdates such as silicomolybdate, phosphomolybdate, and sulfomolybdate [52,59]. This observation has been reiterated and rediscussed in several of our previous publications [45,46,48].

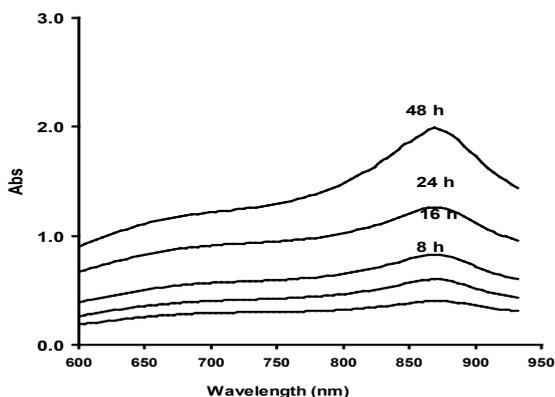


Fig. 1. Scanning absorption spectrum of Mo-blue from *Bacillus* sp. strain Neni-8 at different time intervals.

#### Effect of pH and temperature on molybdate reduction

*Bacillus* sp. strain Neni-8 showed an optimum pH of between 6.3 and 6.5 (Fig. 2) in supporting optimal reduction, while the optimum temperature ranged from 30 °C to 37°C (Fig. 3). The optimum range of temperature will be an advantage for bioremediation works in a tropical region like Sumatera. The majority of the reducers require optimal temperature of between 25 and 37 °C with the only cold-tolerant Mo-reducing bacterium isolated from Antarctica [42] having optimum temperature below 20 °C.

All of the Mo-reducing bacteria isolated to date show optimal pH around subneutral pHs ranging from pH 5.0 to 7.0 [42]. This is probably because acidic pHs play an important role in the formation and stability of phosphomolybdate, and coupled with optimal bacterial metabolic activity needed in reducing molybdenum [54].

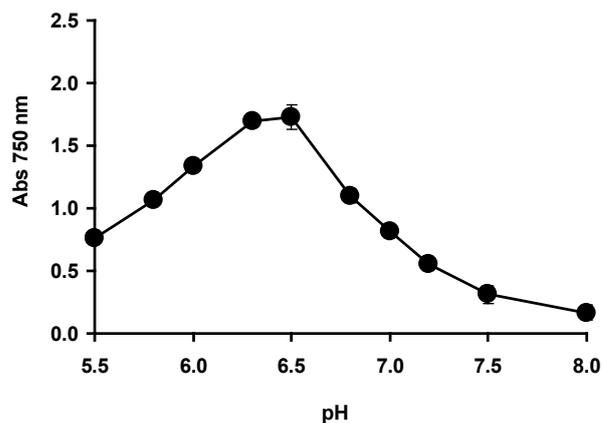


Fig. 2. Molybdenum reduction at various pHs by *Bacillus* sp. strain Neni-8. The error bars are mean  $\pm$  standard deviation of triplicate experiments.

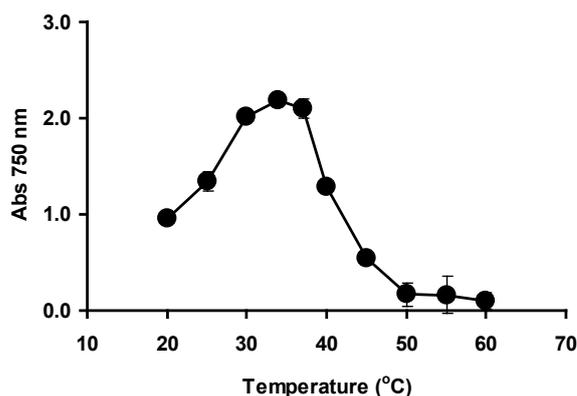


Fig. 3. Molybdenum reduction at various temperatures by *Bacillus* sp. strain Neni-8. The error bars are mean  $\pm$  standard deviation of triplicate experiments.

#### Molybdate reduction utilizing various electron donor

D-glucose was the most optimal electron donor followed in descending order by sucrose, fructose, maltose, lactose, l-arabinose, cellobiose, glycerol, meso-inositol, d-mannose, mannitol, melibiose and sorbitol (Fig. 4). The optimal concentration was 1% (w/v) (data not shown). Most of the Mo-reducing bacteria prefer sucrose or glucose while only *Klebsiella oxytoca* strain hkeem prefers fructose as the most optimal electron donor for reduction [42]. These carbon sources are easily assimilated by bacteria.

With normal metabolic pathways, NADH and NADPH are generated. Incidentally, these compounds are the substrates for the Mo-reducing enzyme [29], thus explaining the preference. A cheaper carbon source for example molasses can be utilized over sucrose and glucose in the future since molasses can be obtained in large quantity as agricultural waste materials especially sugar cane in Malaysia [60]. Molasses has been utilized in bacterial reduction of hexavalent chromate [61,62] and selenate [63]. The effect of molasses as a carbon source is currently being carried out.

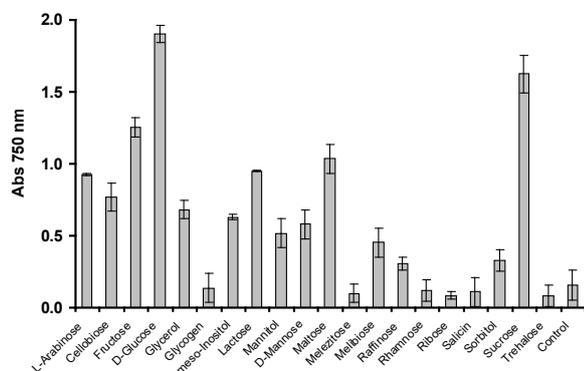


Fig. 4. Molybdenum reduction utilizing various electron donor. The error bars are mean  $\pm$  standard deviation of triplicate experiments.

#### Molybdenum reduction under various concentrations of phosphate and molybdate

Phosphate concentrations affect molybdenum generation, with greater phosphate concentrations drastically reducing output during bacterial molybdenum reduction. Phosphate concentrations larger than 2.9 mM, for example, do limit reduction, according to Ghani *et al.* [18] while concentrations higher than 5 mM inhibited the majority of the Mo-reducing bacteria isolated to date [42]. Phosphate concentrations of 2.5 to 7.5 mM were necessary for optimum reduction, whereas amounts greater than 20 mM severely hindered it (Fig. 5). Glenn and Crane [64] and Shukor *et al.* [65] have shown that high phosphate concentrations disrupt the phosphomolybdate structure by keeping the pH constant at neutral. The phosphomolybdate complex is only stable in acidic environments, and at this pH, it is very unstable [66].

Concentrations of sodium molybdate between 20 and 30 mM were required for optimal reduction, while concentrations higher than 50 mM strongly inhibited reduction (Fig. 6). The bulk of previously identified Mo-reducing bacteria require molybdate concentrations between 5 and 80 mM to support reduction. Because these bacteria can reduce molybdenum at such high quantities, it's safe to assume that molybdenum isn't harmful to them. Molybdenum concentrations in water and soils can be as high as 900 mg/L and 6,500 mg/kg, respectively [5], these bacteria are good candidates for bioremediation of soils and water bodies contaminated with high concentrations of molybdenum. Another important requirement is that soil phosphate concentrations should not exceed 20 mM as this will severely inhibited molybdenum reduction. Since phosphate concentrations rarely exceeded this value in many types of soils, molybdate reduction should proceed unhampered [67].

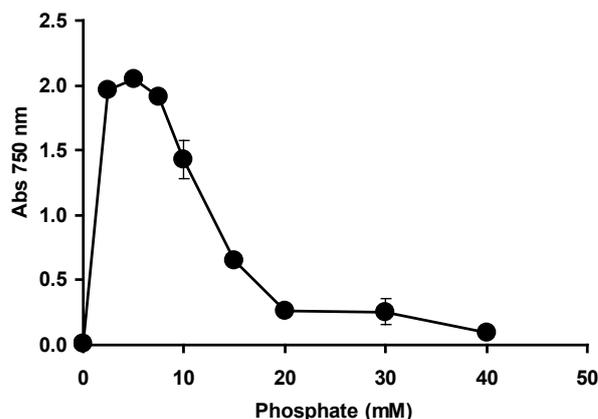


Fig. 5. Mo-blue production under various phosphate concentrations. The error bars are mean  $\pm$  standard deviation of triplicate experiments.

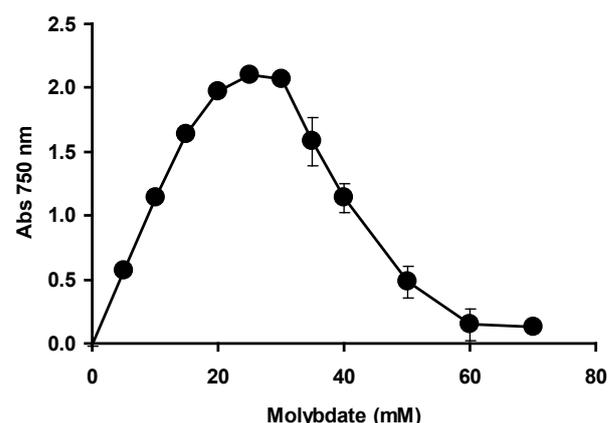
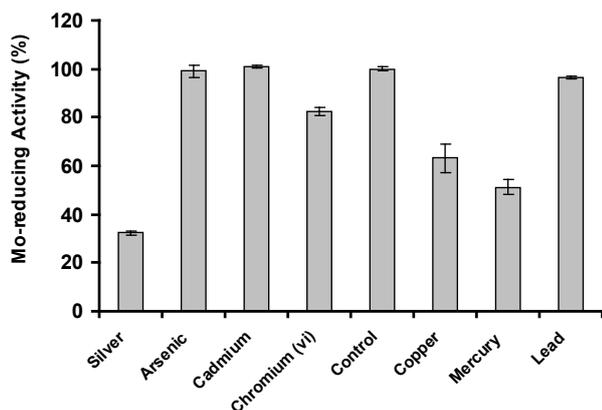


Fig. 6. Mo-blue production under various sodium molybdate concentrations. The error bars are mean  $\pm$  standard deviation of triplicate experiments.

#### Inhibitory effects of heavy metals on molybdate reduction

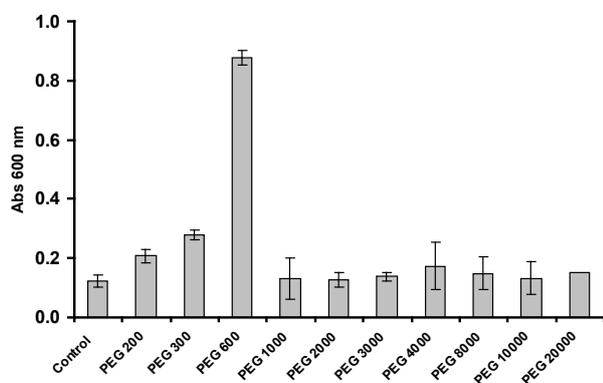
Molybdenum reduction was inhibited by silver, mercury, copper and chromium at 2 ppm by 67.6, 48.7, 36.8 and 17.4 %, respectively (Fig. 7). These heavy metals, especially mercury and copper also inhibit many of the Mo-reducing bacteria isolate to date [42]. Both mercury and copper are also inhibitors of chromate reduction in several bacteria, which include *Bacillus* sp. and *Enterobacter cloacae* strain H01. The sulfhydryl group is the main inhibitory site for heavy metals [68–70]. Metal-sequestering or chelating chemicals such as calcium carbonate, manganese oxide, phosphate, and magnesium hydroxide are used to decrease the harmful effects of heavy metals on other metals. Nonetheless, due to the risk of inhibiting the target metal reduction process, these chemicals must be used with caution. Immobilization of the Mo-reducing bacteria in a membrane, such as dialysis tubing, is another way to shield molybdenum reduction from harmful heavy metals.



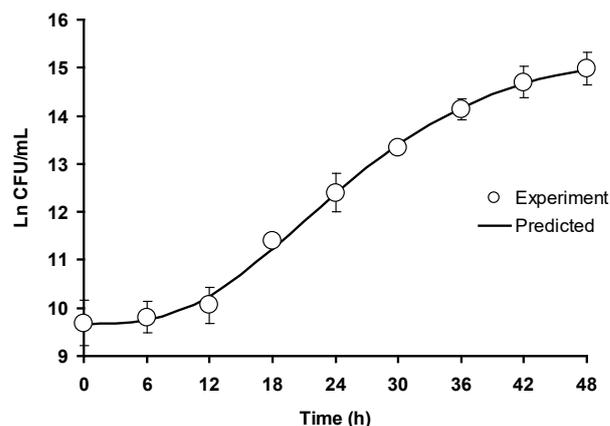
**Fig. 7.** Inhibitory effects of selected heavy metals on Mo-blue production by *Bacillus* sp. strain Neni-8. The error bars are mean  $\pm$  standard deviation of triplicate experiments.

### PEGs as substrates for growth

The bacterium was able to grow on various PEGs such as 200, 300 and 600 (**Fig. 8**). The first PEG-degrading and molybdenum reducing bacterium is *Pseudomonas putida* strain Egypt-15, which is able to grow on PEG 4000 [71]. The growth of this bacterium was modelled according to the modified Gompertz model on PEG 600 as the best carbon source (**Fig. 9**). The growth parameters obtained were theoretical maximum growth of 15.4 ln CFU/ml, a maximum specific growth rate of 0.198 h<sup>-1</sup> and a lag period of 10.1 h. The lag shows that the bacterial cells expend energy to tolerate and activate the metabolic pathways required for PEG absorption. Other microorganisms that have been shown to degrade PEG, including bacteria such as *Pseudomonas solanacearum*, *Alcaligenes xylosoxidans*, *Pseudomonas vesicularis*, and *Enterobacter diversus* [72], *Stenotrophomonas maltophilia*, *Sphingomonas* sp., *Pseudomonas* sp. and *S. macrogoltabida* [73], *Pseudomonas* spp., *Rhodococcus* spp., *Williamsia* spp., *Mycobacterium* spp. and *Bacillus* spp. [74]. Bacteria with multiple detoxification ability is valuable for bioremediation. However, bacteria showing the ability to reduce heavy metal and degrade xenobiotics at the same time are rarely reported. An example is in chromate reduction coupled with phenol degradation [75].



**Fig. 8.** Growth of *Bacillus* sp. strain Neni-8 on various PEGs. The error bars are mean  $\pm$  standard deviation of triplicate experiments.



**Fig. 9.** Growth of *Bacillus* sp. strain Neni-8 on PEG 600 modelled using the modified Gompertz model. The error bars are mean  $\pm$  standard deviation of triplicate experiments.

### CONCLUSION

A Mo-reducing bacterium with the novel ability to use several polyethylene glycol compounds such as PEGs 200, 300 and 600 was reported. The bacterium requires a narrow pH of between 6.3 and 6.5, and temperatures of between 30 and 37 °C for optimal Mo-blue production. The easily assimilable D-glucose was the best electron donor. The bacterium also required a narrow range of phosphate concentrations of between 2.5 and 7.5 mM for optimal activity. The absorption spectrum of the resultant Mo-blue suggests its identity as a reduced phosphomolybdate. The heavy metals silver, mercury, copper and chromium inhibited molybdenum reduction to Mo-blue. The bacterium was tentatively identified as *Bacillus* sp. strain Neni-8. The growth parameters using PEG 600 as the carbon source obtained from the modified Gompertz model showed the presence of a lag period indicating that the bacterial cells spend energy to tolerate and activate metabolic pathways needed for PEG assimilation. Currently, work is underway to characterize PEG-degrading capacity of the bacterium as well as purifying and characterizing the molybdenum-reducing enzyme from this bacterium.

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