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Isolation and Characterization of a Molybdenum-reducing *Bacillus amyloliquefaciens* strain KIK-12 in Soils from Nigeria with the Ability to grow on SDS

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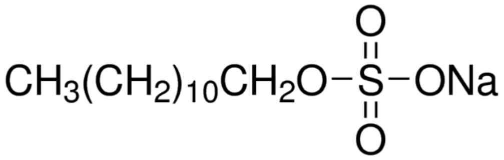
ABSTRACT

The annual production of chemical toxins and organic pollutants has reached an alarming level. Their eradication from the environment is immensely needed, and bioremediation provides a better alternative for this task. In this study, the ability of molybdenum-reducing bacterium isolated from polluted soil to grow and reduce molybdenum on a variety of hydrocarbons and detergents was investigated. The bacterium was found to reduce molybdate to molybdenum blue at an optimum temperature between 25 and 34 °C, pH between 5.8 and 6.3, molybdate concentration between 30 and 50 mM and phosphate concentration between 5.0 and 7.5 mM. The best electron donor hat support molybdate reduction was glucose, followed by sucrose, fructose, maltose, lactose, l-arabinose, d-mannose, mannitol and cellobiose in decreasing order. The absorption spectrum of the resultant Mo-blue was analogous to that of previous Mo-reducing bacterium and bear resemblance with reduced phosphomolybdate. At 2 ppm mercury (ii), copper (ii) and silver (i) molybdenum reduction was inhibited by 82.4, 61.9 and 47.50%, respectively. Based on the biochemical examination, the bacterium was tentatively identified as *Bacillus amyloliquefaciens* strain KIK-12. The ability of this bacterium to degrade detergent and detoxify molybdenum makes it a vital tool for bioremediation.

INTRODUCTION

Human activities have led to an annual increase in the release of harmful toxins and environmental pollutants, which greatly influence the global burden of diseases. Thus, the removal of this toxic substances through bioremediation is considered as a more cost-effective technique than other physicochemical techniques especially at lower concentrations of the toxicant. Molybdenum is among the essential heavy metals required in trace amount and is said to be toxic to some organisms at higher concentrations [1]. Industrially, it is used as an alloying agent, lubricant (as MoS_2), automobile engine anti-freeze and anticorrosion component in steel. These wide industrial application has resulted in some water pollution cases around the globe like in the Tyrol in Austria, Tokyo Bay and the Black Sea, where molybdenum level reaches hundreds of ppm [2,3], in addition terrestrial pollution in sewage sludge [4]. Molybdenum toxic to ruminant at a very low concentration [5,6], inhibit spermatogenesis and arrest embryogenesis in some organisms such as mice and catfish at levels as low as several ppm [7–10].

In addition to heavy metals, detergents represent another form of pollutant being present as co-contaminants in our water bodies. As anionic surfactants, the effect of detergents to aquatic life is detrimental [11] since they exhibited toxicity at lower concentrations ranging from 0.0025 to 300 mg/L [12]. The toxic effect of detergents toward crustaceans and invertebrates has been documented as a result of the continuous release of anionic surfactants into the water bodies. For instance, a study indicated the detrimental effect of SDS on the digestive gland of oyster such as perturbation of the metabolic and nutritional functions [13]. Detergents could also modify the behaviour of the fish such as erratic movement, body torsion and muscle spasms [14].



$$\mathrm{CH_3(CH_2)_{10}CH_2O{-}\overset{O}{\overset{\|}{\underset{\underset{O}{\|}}{S}}}{-}ONa}$$

Fig. 1. The structure of SDS.

Capaldi, and Proskauer were the first to describe the microbial reduction of molybdenum to molybdenum blue more than one century ago in 1896 [15]. This is followed by the works of Jan in 1939 [16], Marchal and Gerard in 1948 [17], Woolfolk and Whiteley in 1962 [18], Bautista and Alexander in 1972 [19], Campbell et al. in 1985 [20], Sugio et al. 1988 (Sugio et al. 1988) and Ghani et al. in 1993 [21] respectively. The work of Campbell et al. on *Escherichia coli* K12 was the first comprehensive studies on bacterial molybdate reduction to Mo-blue. Ghani et al. [21] later identified the potential application of these findings to molybdenum bioremediation.

Recently, more works are being carried out on multi-toxicant remediator microbe, due to the fact that many polluted areas contain numerous toxicants. Reports on heavy metals reduction coupled with xenobiotic degradation are numerous in the literature [22,23]. In a previous study, a molybdenum-reducing bacterium was found to be able to thrive on sodium dodecyl sulfate (SDS) as the only carbon source, even though, SDS does not favor molybdenum reduction [24]. Therefore, finding molybdenum-reducing bacterium capable of xenobiotic degradation is beneficial to bioremediation.

In this study, the potential of the isolated molybdenum-reducing bacterium from contaminated soil to thrive on various hydrocarbons and detergents was investigated, and report on a novel molybdenum-reducing bacterium with the capacity to grow on detergent, SDS. The characteristics of this bacterium make it suitable for future bioremediation works comprising both molybdenum and SDS as an organic pollutant.

MATERIALS AND METHODS

Isolation of a molybdenum-reducing bacterium

In 2014, soil samples were obtained from contaminated land in Kano State, Nigeria. Into a sterile water, one gram of the soil sample was suspended and 0.1 mL aliquot of the suspension was spread onto low phosphate media (LPM) agar compose of Na_2HPO_4 (0.071% or 5 mM), $Na_2MoO_4.2H_2O$ (0.242 % or 10 mM), NaCl (0.5%), $(NH_4)_2.SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), yeast extract (0.5%) and glucose (1%) at pH 7.0 and incubated for 48 hours at room temperature [25].

The formation of blue colonies following incubation signify molybdate reduction. The colony forming the strongest blue color intensity on the LPM agar plate was isolated and restreaked to attain pure culture.

Characterization of molybdenum reduction in liquid media was performed in a shake flask (250 mL) containing 100 mL of LPM media on an orbital shaker (120 rpm) at room temperature for 48 hours. The Mo-blue absorption spectrum was studied by centrifuging 1.0 mL aliquot from the liquid culture at 10,000 × g for 10 minutes at room temperature, after which the supernatant was a scan from 400 to 900 nm using Shimadzu UV-spectrophotometer. A fresh low phosphate media was used as the baseline correction.

Morphological and biochemical characterization of a Mo-reducing bacterium

The bacterium was morphologically and biochemically characterized on the basis of Bergey's manual of determinative bacteriology [26], which include colony shape, size and color on nutrient agar plate, gram staining, motility, oxidase and catalase (24 h), ONPG (beta-galactosidase), ornithine decarboxylase (ODC), arginine dihydrolase (ADH), lysine decarboxylase (LDC), nitrates reduction, Methyl red, indole production, Voges-Proskauer (VP), size and colour on nutrient agar plate, hydrogen sulfide (H_2S), acetate utilization, malonate utilization, citrate utilization (Simmons), esculin hydrolysis, urea hydrolysis, gelatin hydrolysis, phenylalanine deaminase, deoxyribonuclease, lipase (corn oil), acids production from various sugars and gas production from glucose. The result was interpreted via the ABIS online system [27].

Preparation of resting cells for molybdenum reduction characterization

The characterization work such as the effect of temperature, pH, molybdate and phosphate concentrations on molybdenum reduction were statically performed using resting cells in a microtitre plate format as developed previously [28]. Bacterial cells grown overnight in 1 L in high phosphate media (HPM) containing 100 mM on orbital shaker (150 rpm) were harvested by centrifuging at 15,000 × g for 10 minutes, and the pellet was rinsed twice to remove residual phosphate, and then resuspended in 20 mL of LPM not containing glucose to an absorbance reading of 1.00 at 600 nm. An optimal phosphate concentration of 5 mM was uniform to most Mo-reducing bacteria isolated so far.

Thus same concentration was utilized in this work since higher concentration was strongly inhibitory to Mo-blue production [20,21,25,29–41]. Into each well of a sterile microtiter plate, 180 µL was sterically transferred, and 20 µL of glucose (sterile) from a stock solution was added to initiate Mo-blue production. A sterile sealing tape that permits gas exchange (Corning® microplate) was used to seal the plate and incubated at room temperature. Absorbance was measured at time intervals using BioRad microtiter plate reader (Model No. 680) at 750 nm. A specific extinction coefficient of 11.69 $\mathrm{mM.^{-1}.cm^{-1}}$ at 750 nm was used to monitor the Mo-blue production [42].

Effect of heavy metals on molybdenum reduction

Heavy metals namely silver (i), mercury (ii), lead (ii), copper (ii), chromium (vi) cadmium (ii) and arsenic (v) were prepared from Atomic Absorption Spectrometry standard solutions (MERCK) or their commercial salts. The bacterium was incubated with heavy metals in the microplate format at various concentrations. The amount of Mo-blue produced was measured at 750 nm.

Screening of molybdenum reduction using detergents and hydrocarbon as a source of electron donors and for growth

The potential use of hydrocarbons and detergents such as sodium dodecyl benzene sulfonate (SDBS) and sodium dodecyl sulfate (SDS) as carbon source for bacterial growth and as electron donor source to support molybdate reduction was determine by replacing glucose in the LPM with these xenobiotics in a microtiter plate at the final concentration of 200 mg/L for the detergents.

Diesel was first added to 10 mL of the media and sonicated for 5 minutes. The composition of the growth media (LPM) were as follows: $(NH_4)_2.SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), Na_2HPO_4 (0.705% or 50 mM), $NaNO_3$ (0.2%), NaCl (0.5%), yeast extract (0.01%) and 1 mL solution of trace elements containing $CaCl_2$ (40 mg/L), $FeSO_4{\cdot}7H_2O$ (40 mg/L), $MnSO_4{\cdot}4H_2O$ (40 mg/L), $ZnSO_4{\cdot}7H_2O$ (20 mg/L), $CuSO_4{\cdot}5H_2O$ (5 mg/L), $CoCl_2{\cdot}6H_2O$ (5 mg/L), $Na_2MoO_4{\cdot}2H_2O$ (5 mg/L).

The pH of the media was adjusted to 7.0, then 200 uL of the media was transferred into each well of the microtiter plate and incubated at room temperature for 72 hours. The increase in bacterial growth and the amount of Mo-blue produced after 72 hours of incubation were measured at 600 and 750 nm, respectively using microplate reader (Bio-Rad 680).

Statistical analysis

Data analyses were conducted using InStat GraphPad version 3.05 available on www.graphpad.com. One-way analysis of variance with Tukey's post hoc analysis was employed to compare between groups. Results are expressed as mean ± SD with $p<0.05$ considered statistically significant.

RESULTS AND DISCUSSION

The phenomenon of microbial molybdate reduction to Mo-blue was first reported in 1896 by Capaldi and Proskauer in *E. coli* [15]. Although, further reports on the phenomenon are sparse, however, the work [20] on *Escherichia coli* K12 came as the earliest comprehensive report describing the phenomenon of molybdate reduction to Mo-blue in bacteria. Much later, [21] recognize the potential application of this microbiological phenomenon to bioremediation of molybdenum.

All of the earlier works deal with molybdenum reduction and not in association with degradation of other toxicants. Thus, the isolation of more new molybdenum-reducers with the potential to degrade other toxicants is advantageous to bioremediation.

Identification of molybdenum-reducing bacterium

The morphological characteristic shows that the bacterium is a Gram-positive, rod-shaped, while comparing the result of the biochemical tests with Bergey's manual of determinative bacteriology [26] using the ABIS online software [27] gave three suggestions to the bacterial identity, in which the highest homology of 86% and accuracy of 88% was related to *Bacillus amyloliquefaciens*. Although, more molecular studies like 16S rRNA may be required in the future to further identify this species. However, at this point, the bacterium was tentatively identified as *Bacillus amyloliquefaciens* strain KIK-12.

Table 1. Biochemical tests for *Bacillus amyloliquefaciens* strain KIK-12.

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

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

Previously, two other molybdenum-reducers from this genus, *Bacillus* sp. strain A.rzi [40] and *Bacillus pumilus* strain lbna [37] have been isolated and characterized. The characterization work of this bacterium involves the use of a rapid, simple and throughput method of microplate format to speed up work and obtain more data than the usual shake-flask approach [28,43]. Similarly, some bacteria from this genus have been reported to degrade and utilize detergent including SDS [44–48] as a carbon source for growth. The use of resting cells under the static condition to characterize bacterial molybdate reduction was begun by [21]. Resting cells were also used to study heavy metals reduction such as selenate [49] and detergent like SDS [50].

Molybdenum-blue absorption spectrum

Periodic scanning of Mo-blue absorption spectrum from this bacterium reveals similarity to that of previous isolates, with a maximum peak between 860 and 870 nm, and a shoulder close to 700 nm. The intensity of the Mo-blue increases from 8 to 48 hours with the preservation of the characteristics fingerprint profile (**Fig. 2**).

It has been suggested earlier by [20] that the Mo-blue observed during molybdate reduction in *E. coli* K12 is a reduced form of phosphomolybdate due to its similarity with the Mo-blue formed in the phosphate determination method. In the phosphate determination method, Mo-blue absorption spectrum showed a maximum peak around 880 to 890 nm and a shoulder near 700 to 720 nm [34]. Although, the selected wavelength in this work (750 nm) has approximately 30% lower intensity than at 865 nm, but is enough for routine monitoring of the Mo-blue production since it is high enough not to be masked by cellular absorption between 600-620 nm similar to previous results [28].

It was generally observed that the entire Mo-blue spectra from other bacteria show the same characteristics, which imply the involvement of probably same Mo-blue species [29]. Perhaps, the exact identification of the phosphomolybdate species require the use of NMR and ESR [51], however, scanning the spectroscopic profile is enough and acceptable means of characterizing the heteropolymolybdate species [52]. Previous monitoring of Mo-blue production uses 820 nm [20] and 710 nm [21], both of which were claimed to have low interference by bacterial cellular absorption. Mo-blue has a complex structure with mixed valency in the oxidation state (between 5+ and 6+) of many species [29,51], making it precise identification of the species difficult.

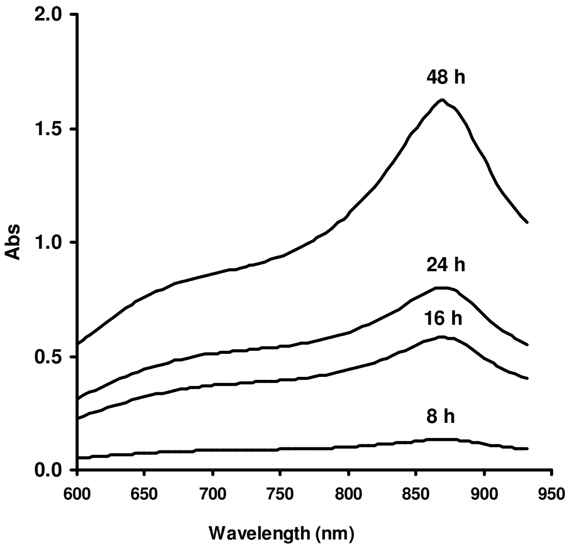


Fig. 2 Periodic scanning of Mo-blue absorption spectrum from *Bacillus amyloliquefaciens* strain KIK-12.

Effect of pH and temperature on molybdenum reduction

Analysis of variance shows that the optimum pH supporting molybdenum reduction in this bacterium was between 6.0 and 6.5, after incubation of *Bacillus amyloliquefaciens* strain KIK-12 in 20 mM Bis-Tris and Tris.Cl buffers, pH ranging from 5.5 to 8.0. Mo-blue production was dramatically inhibited at pH lower than 5 (**Fig. 3**).

Similarly, studying the effect of temperature over a wide range (20 to 60 °C) reveals no significant difference (p>0.05) among the values, with optimum temperature occurring between 30 °C to 37°C (**Fig. 4**). However, a temperature lower than 30 °C and higher than 37 °C strongly lower Mo-blue production by *Bacillus amyloliquefaciens* strain KIK-12.

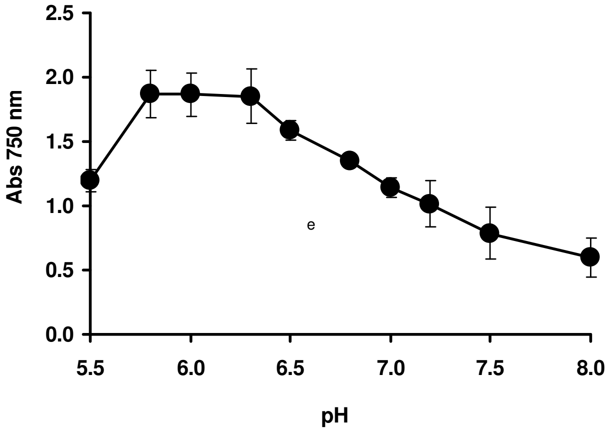


Fig. 3. Effect of pH on molybdenum-blue production by *Bacillus amyloliquefaciens* strain KIK-12, after 48 hours incubation of resting cells in a microtiter plate under optimized conditions. The error bars represent the mean ± SD of triplicate.

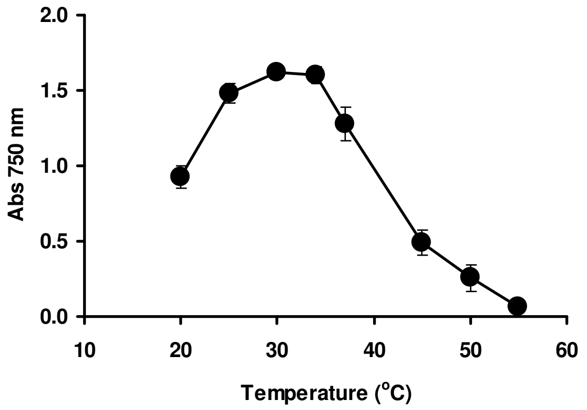


Fig. 4. Effect of temperature on molybdenum-blue production by *Bacillus amyloliquefaciens* strain KIK-12, after 48 hours incubation of resting cells in a microtiter plate under optimized conditions. The error bars represent the mean ± SD of triplicate.

The pH and temperature play an important role in enzyme-catalyzed molybdate reduction, as both parameters affect enzymatic activity by altering with the ionic character of the amino acids constituent and hence the tertiary and quaternary protein structure and folding. The optimum conditions obtained in this work would be of advantage to molybdenum bioremediation in a tropical country like Nigeria, having an annual average temperature ranging from 25 to 30 °C. The greatest majority of Mo-reducers show an optimal temperature of between 25 and 37 °C [25,30,31,33–37,39–41,53] since they are isolated from tropical soils, with the only exception being the psychrotolerant reducer isolated from Antarctic region showing an optimal temperature between 15 and 20 °C to support Mo-blue production [38].

Bacillus amyloliquefaciens strain KIK-12 shows characteristics of a neutrophile in supporting molybdenum reduction. A typical neutrophile grows best at pH between 5.5 and 8.0. A general observation regarding bacterial molybdenum reduction is that the optimal pH for the reduction process is slightly acidic, optimally ranging from pH 5.0 to 7.0 [20,21,30–41,53]. It has been earlier suggested that acidic pH is necessary for the formation and stability of phosphomolybdate intermediate before being reduced to Mo-blue. Thus, optimal reduction requires balancing between substrate stability and enzyme activity [29].

Effect of electron donor on molybdate reduction

Previous studies have demonstrated that some Mo-reducing bacteria like E*scherichia coli* K12 [20], *Serratia* sp. strain Dr.Y5 [31], *Enterobacter* sp. strain Dr.Y13 [32], *Pseudomonas* sp. strain DRY2 [34], *Acinetobacter calcoaceticus* strain Dr.Y12 [35], *Pseudomonas* sp. strain DRY1 [38], *Bacillus pumilus* strain lbna [37] and *Bacillus* sp. strain A.rzi [40] prefer glucose as most suitable carbon source, whereas, *Enterobacter cloacae* strain 48 [21], *Serratia marcescens* strain DRY6 [30], *Serratia* sp. strain Dr.Y5 [31] and *S. marcescens* strain Dr.Y9 [25] showed sucrose as the best carbon source, and *Klebsiella oxyto*ca strain hkeem prefers fructose [36].

In the present study, glucose was the best electron donor source supporting molybdate reduction followed by sucrose, d-mannose, maltose, d-sorbitol, trehalose, d-mannitol, glycerol, salicin and myo-inositol (**Fig. 5**). Microorganism metabolize carbon sources in the media to produce electron donating substrates, NADH and NADPH, which are responsible for donating electron during molybdenum reduction [53,54]. Glucose and sucrose easily metabolized to generate these reducing equivalents (NADH and NADPH) in chromate reduction by chromate reductase in some bacteria [55–57].

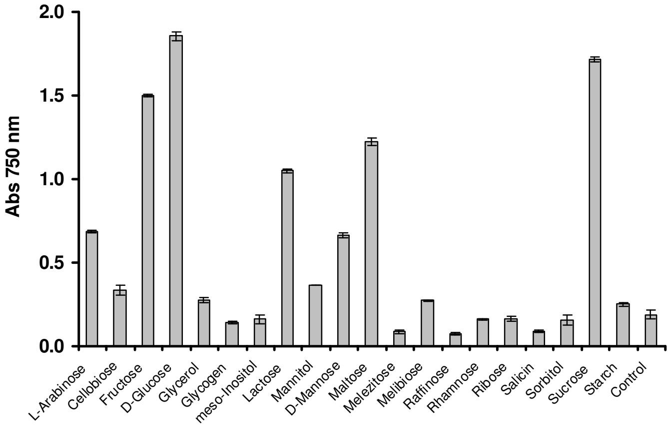


Fig. 5. Growth of *Bacillus amyloliquefaciens* strain KIK-12 in 10 mM low phosphate media containing 1% (w/v) of various electron donor sources. The resting cells were incubated in a microtiter plate for 48 hours under optimized condition. The error bars represent the mean ± SD of triplicate.

Effect of molybdate and phosphate concentrations on molybdenum-blue production

The optimum phosphate concentration supporting Mo-reduction in this bacterium occurred between 5.0 and 7.5 mM, with higher concentrations being inhibitory to Mo-blue production (**Fig. 6**). The strong buffering power of the phosphate buffer occurring at high phosphate concentration is suggested to inhibit the stability of phosphomolybdate complex which requires an acidic condition for its formation [58–60].

All molybdenum-reducing bacterium isolated so far require phosphate concentration not higher than 5 mM for optimal reduction [20,21,30–41,53]. Determining the optimal phosphate and molybdate concentrations supporting Mo-blue production is important since both anions have been shown to inhibit the reduction process in bacteria [25,30,32–36,38,40,53].

The current study revealed that the newly isolated bacterium was able to tolerate and reduce as high as 60 mM molybdate to Mo-blue, although optimal reduction occurred between 30 and 50 mM (**Fig. 7**). The reduction of this high concentration of molybdate to an insoluble form would enable the strain to detoxify high molybdenum pollution. *Pseudomonas* sp strain Dr.Y2 was reported to reduce the lowest optimal concentration of molybdenum at 15 mM [34]. However, *E. coli* K12 [20] and *Klebsiella oxytoca* strain hkeem [36] were reported to optimally reduce the highest molybdenum at 80 mM.

Other Mo-reducers such as EC48 [21], *Serratia marcescens* strain Dr.Y6 [30], *Serratia marcescens*. Dr.Y9 [25], *Pseudomonas* sp. strain Dr.Y2 [34], *Serratia* sp. strain Dr.Y5 [31], *Enterobacter* sp. strain Dr.Y13 [32] and *Acinetobacter calcoaceticus* [35] produce optimal Mo-blue at optimal molybdate concentrations of 50, 25, 55, 30, 30, 50 and 20 mM, respectively. To date, the highest concentration of molybdenum reported as a pollutant in the environment is around 2000 ppm or about 20 mM [61].

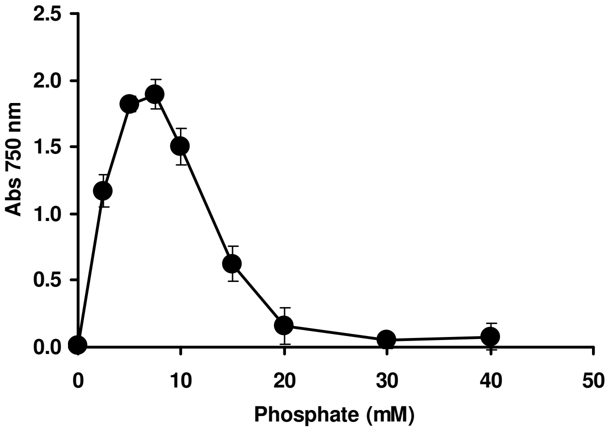


Fig. 6. Effect of phosphate concentrations on molybdenum-blue production by *Bacillus amyloliquefaciens* strain KIK-12, after 48 hours incubation of resting cells in a microtiter plate under optimized conditions. The error bars represent the mean ± SD of triplicate.

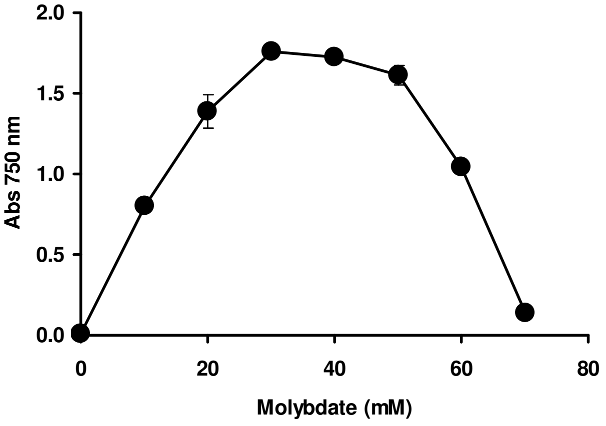


Fig. 7. Effect of molybdate concentrations on molybdenum-blue production by *Bacillus amyloliquefaciens* strain KIK-12, after 48 hours incubation of resting cells in a microtiter plate under optimized conditions. The error bars represent the mean ± SD of triplicate.

Effect of heavy metals

Molybdenum reduction in strain KIK-12 was found to be inhibited at 2 ppm mercury (ii), copper (ii) and silver (i) by 82.4, 61.9 and 47.50%, respectively (**Fig. 8**). The inhibitory effect by other interacting metal ions, present a major challenge to the success of bioremediation. Therefore, it is of importance to screen and isolate more bacteria with as many metal tolerances as possible. Heavy metals such as cadmium, copper, mercury and silver usually target the sulfhydryl group of proteins [62]. Mercury is a physiological inhibitor to molybdate reduction [63], while chromate is known to inhibit certain enzymes like glucose oxidase [64] and enzymes of nitrogen metabolism in plants [65].

The binding of heavy metals inactivated metal-reducing capacity of the enzyme(s) responsible for the reduction process. A summary of the type of heavy metals that inhibited Mo-reducing bacteria showed that almost all of the reducers are inhibited by toxic heavy metals (**Table 2**).

Table 2. Heavy metals inhibition of Mo-reducing bacteria.

Bacteria	Heavy Metals that inhibit reduction	Author
Bacillus pumilus strain lbna	As^{3+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	[37]
Bacillus sp. strain A.rzi	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, $Hg^{2+,}$ Co^{2+},Zn^{2+}	[40]
Serratia sp. strain Dr.Y8	Cr, Cu, Ag, Hg	[33]
S. marcescens strain Dr.Y9	Cr^{6+}, Cu^{2+}, Ag^{+}, Hg^{2+}	[25]
Serratia sp. strain Dr.Y5	n.a.	[31]
Pseudomonas sp. strain DRY2	Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	[34]
Pseudomonas sp. strain DRY1	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, Hg^{2+}	[38]
Enterobacter sp. strain Dr.Y13	Cr^{6+}, Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}	[32]
Acinetobacter calcoaceticus strain Dr.Y12	Cd^{2+}, Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	[35]
Serratia marcescens strain DRY6	Cr^{6+}, Cu^{2+}, Hg^{2+}*	[30]
Enterobacter cloacae strain 48	Cr^{6+}, Cu^{2+}	[21]
Escherichia coli	Cr^{6+}	[20]
Klebsiella oxytoca strain hkeem	Cu^{2+}, Ag^{+}, Hg^{2+}	[36]

Hydrocarbon and detergents as a carbon source for growth and electron donor sources for molybdenum reduction

Screening of hydrocarbons and detergents as electron donor sources supporting Mo-blue production failed to give a positive result, even though, the bacterium was able to grow well on the detergent SDS (**Fig.** 9). SDS-degrading bacteria are more ideal for SDS remediation due to their economic benefits over the physicochemical methods. Microbes are known for their ability to degrade organic compounds, thus use as bioremediation agents for economical removal of xenobiotic pollutants [66]. Biodegradation of anionic surfactant under aerobic condition by *Pseudomonas* sp. strain C12B was among the first to be studied [67]. To date, quite numerous SDS-degrading bacteria have been isolated and characterized [24,66,68–72]. Perhaps, studies on cold-tolerant microbes with the ability to degrade SDS are rare and was first reported in 1998 by Margesin and Schinner [73,74]. The existence of a multitude of bacteria with detergent- and hydrocarbon-degrading ability makes bioremediation the ideal method for detergent degradation.

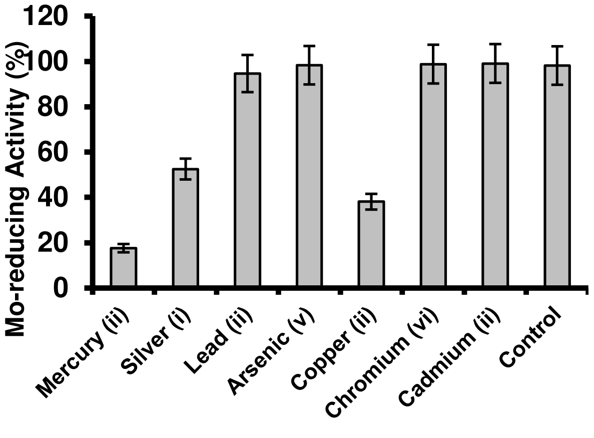


Fig. 8. Effect of metal ions on Mo-blue production by *Bacillus amyloliquefaciens* strain KIK-12, after 48 hours incubation of resting cells in a microtiter plate under optimized conditions. The error bars represent the mean ± SD of triplicate.

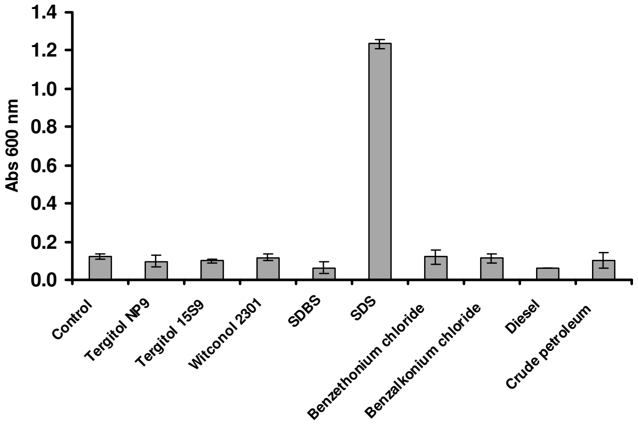


Fig. 9. Growth of *Bacillus amyloliquefaciens* strain KIK-12 resting cells, after 48 hours incubation on various xenobiotics in a microtiter plate under optimized conditions. The error bars represent the mean ± SD of triplicate.

CONCLUSION

A locally isolated Mo-reducing bacterium with novel ability to degrade SDS is reported. The bacterium produced Mo-blue optimally at pH between 5.8 and 6.3, temperature between 25 and 34 ºC, phosphate and molybdate concentrations between 5.0 and 7.5 mM and between 30 and 50 mM respectively. Glucose was the best electron donor source supporting molybdate reduction followed by sucrose, fructose, maltose, lactose, l-arabinose, d-mannose, mannitol and cellobiose. The Mo-blue absorption spectrum was similar to that of the previous Mo-reducing bacterium and closely resembles a reduced phosphomolybdate species. Molybdenum reduction in this bacterium was inhibited at 2 ppm mercury (ii), copper (ii) and silver (i) by 82.4, 61.9 and 47.50%, respectively. The ability of strain KIK-12 to degrade xenobiotics and simultaneously reduce heavy metals like molybdenum will make it useful for bioremediation of co-contaminated sites. Currently, work is ongoing to purify molybdenum-reducing enzyme from this bacterium and characterize the detergent degradation ability in more detail.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Abo-Shakeer LKA, Ahmad SA, Shukor MY, et al (2013) Isolation and characterization of a molybdenum-reducing *Bacillus pumilus* strain lbna. J Environ Microbiol Toxicol 1:9–14.
2. AhmadPanahi H, Hosseinzadeh M, Adinehlo H, et al (2014) Removal of molybdenum from environmental sample by adsorption using modified aniline-formaldehyde with salicylic acid. World Appl Sci J 30:1892–1898.
3. Ahmad SA, Shukor MY, Shamaan NA, et al (2013) Molybdate reduction to molybdenum blue by an antarctic bacterium. BioMed Res Int. doi: 10.1155/2013/871941
4. Amund OO, Ilori MO, Odetundun FR (1997) Degradation of commercial detergent products by microbial populations of the Lagos Lagoon. Folia Microbiol (Praha) 42:353–356.
5. Bautista EM, Alexander M (1972) Reduction of inorganic compounds by soil microorganisms. Soil Sci Soc Am Proc 36:918–920.
6. Bi C-M, Zhang Y-L, Liu F-J, et al (2013) The effect of molybdenum on the in vitro development of mouse preimplantation embryos. Syst Biol Reprod Med 59:69–73. doi: 10.3109/19396368.2012.743198
7. Campbell AM, Del Campillo-Campbell A, Villaret DB (1985) Molybdate reduction by *Escherichia coli* K-12 and its chl mutants. Proc Natl Acad Sci U S A 82:227–231.
8. Capaldi A, Proskauer B (1896) Beiträge zur Kenntniss der Säurebildung bei Typhus-bacillen und Bacterium coli - Eine differential-diagnostische Studie. Z Für Hyg Infect 23:452–474.
9. Chaturvedi V, Kumar A (2011a) Diversity of culturable sodium dodecyl sulfate (SDS) degrading bacteria isolated from detergent contaminated ponds situated in Varanasi city, India. Int Biodeterior Biodegrad 65:961–971. doi: 10.1016/j.ibiod.2011.07.005
10. Chaturvedi V, Kumar A (2011b) Isolation of a strain of *Pseudomonas putida* capable of metabolizing anionic detergent sodium dodecyl sulfate (SDS). Iran J Microbiol 3:47–53.
11. Chirwa EN, Wang Y-T (2000) Simultaneous chromium(VI) reduction and phenol degradation in an anaerobic consortium of bacteria. Water Res 34:2376–2384.
12. Chung J, Rittmann BE, Wright WF, Bowman RH (2007) Simultaneous bio-reduction of nitrate, perchlorate, selenate, chromate, arsenate, and dibromochloropropane using a hydrogen-based membrane biofilm reactor. Biodegradation 18:199–209.
13. Costin S, Ionut S (2015) ABIS online - bacterial identification software, http://www.tgw1916.net/bacteria_logare.html, database version: Bacillus 022012-2.10, accessed on Mar 2015.
14. Cserháti T, Forgács E, Oros G (2002) Biological activity and environmental impact of anionic surfactants. Environ Int 28:337–348. doi: 10.1016/S0160-4120(02)00032-6
15. Davis GK (1991) Molybdenum. In: Merian E (ed) Metals and their Compounds in the Environment, Occurrence, Analysis and Biological Relevance. VCH Weinheim, New York, pp 1089–1100
16. Elangovan R., Philip L., Chandraraj K. (2010) Hexavalent chromium reduction by free and immobilized cell-free extract of *Arthrobacter rhombi*-RE. Appl Biochem Biotechnol 160:81–97.
17. Ghani B, Takai M, Hisham NZ, et al (1993) Isolation and characterization of a Mo^{6+}-reducing bacterium. Appl Environ Microbiol 59:1176–1180.
18. Glenn JL, Crane FL (1956) Studies on metalloflavoproteins. V. The action of silicomolybdate in the reduction of cytochrome c by aldehyde oxidase. Biochim Biophys Acta 22:111–115.
19. Halmi MIE, Hussin WSW, Aqlima A, et al (2013a) Characterization of a sodium dodecyl sulphate-degrading *Pseudomonas* sp. strain DRY15 from Antarctic soil. J Environ Biol 34:1077–1082.
20. Halmi MIE, Zuhainis SW, Yusof MT, et al (2013b) Hexavalent molybdenum reduction to Mo-blue by a sodium-dodecyl-sulfate-degrading *Klebsiella oxytoca* strain dry14.
21. Halmi MIE, Zuhainis SW, Yusof MT, et al (2013c) Hexavalent molybdenum reduction to Mo-blue by a Sodium-Dodecyl-Sulfate-degrading *Klebsiella oxytoca* strain DRY14. BioMed Res Int 2013:e384541. doi: 10.1155/2013/384541
22. Holt JG, Krieg NR, Sneath PHA, et al (1994) Bergey's Manual of Determinative Bacteriology, 9th edn. Lippincott Williams & Wilkins
23. Iyamu EW, Asakura T, Woods GM (2008) A colorimetric microplate assay method for high-throughput analysis of arginase activity in vitro. Anal Biochem 383:332–334.
24. Jan A (1939) La reduction biologique du molybdate d'ammonium par les bactéries du genre Serratia (The biological reduction of ammonium molybdate by the bacteria of the Serratia kind). Bull Sci Pharmacol 46:336–339.
25. Kazansky LP, Fedotov MA (1980) Phosphorus-31 and oxygen-17 N.M.R. evidence of trapped electrons in reduced 18-molybdodiphosphate(V), $P_2Mo_{18}O_{62}$ 8-. J Chem Soc Chem Commun 644–646.
26. Khan A, Halmi MIE, Shukor MY (2014) Isolation of Mo-reducing bacterium in soils from Pakistan. J Environ Microbiol Toxicol 2:38–41.
27. Kincaid RL (1980) Toxicity of ammonium molybdate added to drinking water of calves. J Dairy Sci 63:608–610.
28. Lahann RW (1976) Molybdenum hazard in land disposal of sewage sludge. Water Air Soil Pollut 6:3–8.
29. Lim HK, Syed MA, Shukor MY (2012) Reduction of molybdate to molybdenum blue by *Klebsiella* sp. strain hkeem. J Basic Microbiol 52:296–305. doi: 10.1002/jobm.201100121
30. Liwarska-Bizukojc E., Miksch K., Malachowska-Jutsz A., Kalka J. (2005) Acute toxicity and genotoxicity of five selected anionic and nonionic surfactants. Chemosphere 58:1249–1253.
31. Losi ME, Jr WTF (1997) Reduction of selenium oxyanions by *Enterobacter cloacae* strain SLD1a-1: Reduction of selenate to selenite. Environ Toxicol Chem 16:1851–1858.
32. Marchal JG, Gerard TH (1948) Etude du pouvoir reducteur de quelque souches de colibacille sur le molybdate d'ammoniaque. Trav Lab Microbiol Fac Pharm Nancy 16:11–23.
33. Margesin R, Schinner F (1998) Biodegradation of the anionic surfactant sodium dodecyl sulfate at low temperatures. Int Biodeterior Biodegrad 41:139–143. doi: 10.1016/S0964-8305(97)00084-X
34. Meeker JD, Rossano MG, Protas B, et al (2008) Cadmium, lead, and other metals in relation to semen quality: Human evidence for molybdenum as a male reproductive toxicant. Environ Health Perspect 116:1473–1479.
35. Neunhäuserer C, Berreck M, Insam H (2001) Remediation of soils contaminated with molybdenum using soil amendments and phytoremediation. 128:85–96.
36. Ogbulie TE., Ogbulie JN., Umezuruike I. (2008) Biodegradation of detergents by aquatic bacterial flora from Otamiri River, Nigeria. Afr J Biotechnol 7:824–830.
37. Okpokwasili GC, Olisa AO (1991) River-water biodegradation of surfactants in liquid detergents and shampoos. Water Res 25:1425–1429. doi: 10.1016/0043-1354(91)90121-6
38. Opperman DJ, Piater LA, Van Heerden E (2008) A novel chromate reductase from *Thermus scotoductus* SA-01 related to old yellow enzyme. J Bacteriol 190:3076–3082.
39. Othman AR, Bakar NA, Halmi MIE, et al (2013) Kinetics of molybdenum reduction to molybdenum blue by *Bacillus* sp. strain A.rzi. BioMed Res Int. doi: 10.1155/2013/371058
40. Payne WJ, Feisal VE (1963) Bacterial utilization of dodecyl sulfate and dodecyl benzene sulfonate. Appl Microbiol 11:339–344.
41. Pettersson A, Adamsson M, Dave G (2000) Toxicity and detoxification of Swedish detergents and softener products. Chemosphere 41:1611–1620. doi: 10.1016/S0045-6535(00)00035-7

42. Rahman MFA, Shukor MY, Suhaili Z, et al (2009) Reduction of Mo(VI) by the bacterium *Serratia* sp. strain DRY5. J Environ Biol 30:65–72.
43. Rosety M, Ribelles A, Rosety-Rodriguez M, et al (2000) Morpho-histochemical study of the biological effects of sodium dodecyl sulphate on the digestive gland of the Portuguese oyster. Histol Histopathol 15:1137–1143.
44. Runnells DD, Kaback DS, Thurman EM (1976) Geochemistry and sampling of molybdenum in sediments, soils, and plants in Colorado. In: Chappel WR, Peterson KK (eds) Molybdenum in the environment. Marcel and Dekker, Inc., New York,
45. Sangwan P, Kumar V, Joshi UN (2014) Effect of chromium(VI) toxicity on enzymes of nitrogen metabolism in clusterbean (*Cyamopsis tetragonoloba* L.). Enzyme Res 2014:784036.
46. Shen H, Wang Y-T (1994) Biological reduction of chromium by *E. coli*. J Environ Eng 120:560–572.
47. Shukor MS, Shukor MY (2014) A microplate format for characterizing the growth of molybdenum-reducing bacteria. J Environ Microbiol Toxicol 2:42–44.
48. Shukor MY, Ahmad SA, Nadzir MMM, et al (2010a) Molybdate reduction by *Pseudomonas* sp. strain DRY2. J Appl Microbiol 108:2050–2058. doi: 10.1111/j.1365-2672.2009.04604.x
49. Shukor MY, Habib SHM, Rahman MFA, et al (2008a) Hexavalent molybdenum reduction to molybdenum blue by *S. marcescens* strain Dr. Y6. Appl Biochem Biotechnol 149:33–43. doi: 10.1007/s12010-008-8137-z
50. Shukor MY, Halmi MIE, Rahman MFA, et al (2014) Molybdenum reduction to molybdenum blue in *Serratia* sp. strain DRY5 is catalyzed by a novel molybdenum-reducing enzyme. BioMed Res Int. doi: 10.1155/2014/853084
51. Shukor MY, Husin WSW, Rahman MFA, et al (2009a) Isolation and characterization of an SDS-degrading *Klebsiella oxytoca*. J Environ Biol 30:129–134.
52. Shukor MY, Lee CH, Omar I, et al (2003) Isolation and characterization of a molybdenum-reducing enzyme in *Enterobacter cloacae* strain 48. Pertanika J Sci Technol 11:261–272.
53. Shukor MY, Rahman MFA, Shamaan NA, et al (2008b) An improved enzyme assay for molybdenum-reducing activity in bacteria. Appl Biochem Biotechnol 144:293–300.
54. Shukor MY, Rahman MF, Shamaan NA, Syed MS (2009b) Reduction of molybdate to molybdenum blue by *Enterobacter* sp. strain Dr.Y13. J Basic Microbiol 49:S43–S54. doi: 10.1002/jobm.200800312
55. Shukor MY, Rahman MF, Suhaili Z, et al (2009c) Bacterial reduction of hexavalent molybdenum to molybdenum blue. World J Microbiol Biotechnol 25:1225–1234. doi: 10.1007/s11274-009-0006-6
56. Shukor MY, Rahman MF, Suhaili Z, et al (2010b) Hexavalent molybdenum reduction to Mo-blue by *Acinetobacter calcoaceticus*. Folia Microbiol (Praha) 55:137–143. doi: 10.1007/s12223-010-0021-x
57. Shukor MY, Shamaan NA, Syed MA, et al (2000) Characterization and quantification of molybdenum blue production in *Enterobacter cloacae* strain 48 using 12-molybdophosphate as the reference compound. Asia-Pac J Mol Biol Biotechnol 8:167–172.
58. Shukor MY, Syed MA, Lee CH, et al (2002) A method to distinguish between chemical and enzymatic reduction of molybdenum in *Enterobacter cloacae* strain 48. Malays J Biochem 7:71–72.
59. Shukor Y, Adam H, Ithnin K, et al (2007a) Molybdate reduction to molybdenum blue in microbe proceeds via a phosphomolybdate intermediate. J Biol Sci 7:1448–1452.
60. Shukor Y, Adam H, Ithnin K, et al (2007b) Molybdate reduction to molybdenum blue in microbe proceeds via a phosphomolybdate intermediate. 7:1448–1452.
61. Sims RPA (1961) Formation of heteropoly blue by some reduction procedures used in the micro-determination of phosphorus. 86:584–590.
62. Singh KL, Kumar A, Kumar A (1998) Short communication: *Bacillus cereus* capable of degrading SDS shows growth with a variety of detergents. World J Microbiol Biotechnol 14:777–779.
63. Sugiura Y, Hirayama Y (1976) Structural and electronic effects on complex formation of copper(II) and nickel(II) with sulfhydryl-containing peptides. Inorg Chem 15:679–682.
64. Syed MA, Mahamood M, Shukor MY, Shamaan NA (2010) Isolation and characterization of SDS-degrading *Pseudomonas aeruginosa* sp. strain D1. Aust J Basic Appl Sci 4:5000–5011.
65. Underwood EJ (1979) Environmental sources of heavy metals and their toxicity to man and animals. 11:33–45.
66. Usharani J, Rajasekar T, Deivasigamani B (2012) Isolation and characterizatiosn of SDS (Sodium Dodecyl Sulfate) degrading organisms from SDS contaminated areas. Sci Rep 1:148.
67. Venkatesh C (2013) A plate assay method for isolation of bacteria having potent Sodium Dodecyl Sulfate (SDS) degrading ability. Res J Biotechnol 8:27–31.
68. Willetts AJ, Cain RB (1972) Microbial metabolism of alkylbenzene sulphonates Enzyme system of a *Bacillus* sp. responsible for B-oxidation of the alkyl side chain of alkylbenzene sulphonates. Antonie Van Leeuwenhoek 38:543–555. doi: 10.1007/BF02328121
69. Woolfolk CA, Whitely HR (1962) Reduction of inorganic compounds with molecular hydrogen by *Micrococcus lactilyticus* I. Stoichiometry with compounds of arsenic, selenium, tellurium, transition and other elements. J Bacteriol 84:647–658.
70. Yilmaz F, Icgen B (2014) Characterization of SDS-degrading *Delftia acidovorans* and in situ monitoring of its temporal succession in SDS-contaminated surface waters. Environ Sci Pollut Res 21:7413–7424.
71. Yoshimura K, Ishii M, Tarutani T (1986) Microdetermination of phosphate in water by gel-phase colorimetry with molybdenum blue. Anal Chem 58:591–594.
72. Yunus SM, Hamim HM, Anas OM, et al (2009) Mo (VI) reduction to molybdenum blue by *Serratia marcescens* strain Dr. Y9. Pol J Microbiol 58:141–147.
73. Zeng G-M, Tang L, Shen G-L, et al (2004) Determination of trace chromium (VI) by an inhibition-based enzyme biosensor incorporating an electropolymerized aniline membrane and ferrocene as electron transfer mediator. Int J Environ Anal Chem 84:761–774. doi: 10.1080/03067310410001730619
74. Zhai X-W, Zhang Y-L, Qi Q, et al (2013) Effects of molybdenum on sperm quality and testis oxidative stress. Syst Biol Reprod Med 59:251–255. doi: 10.3109/19396368.2013.791347
75. Zhang Y-L, Liu F-J, Chen X-L, et al (2013) Dual effects of molybdenum on mouse oocyte quality and ovarian oxidative stress. Syst Biol Reprod Med 59:312–318. doi: 10.3109/19396368.2013.826296