HIBISCUS PUBLISHER

BIOREMEDIATION SCIENCE AND TECHNOLOGY RESEARCH

Website: http://journal.hibiscuspublisher.com/index.php/BSTR

Isolation and Characterization of a Molybdenum-reducing and Methylene Blue-decolorizing *Serratia marcescens* strain KIK-1 in Soils from Nigeria

Karamba, K.I.[1] and Yakasai H.M[2]*

[1]Department of Biochemistry, Faculty of Basic Medical Sciences, College of Health Science, Bayero University Kano, P. M. B 3011, Kano State-Nigeria.
[2]Department of Microbiology, Bauchi State University, Gadau, Bauchi State, Nigeria.

*Corresponding author:
Hafeez Muhammad Yakasai
Department of Biochemistry,
Faculty of Basic Medical Sciences,
College of Health Sciences,
Bayero University Kano,
P. M. B 3011.
Email: hmyakasai.bch@buk.edu.ng

HISTORY

Received: 27th March 2017
Received in revised form: 2nd May 2017
Accepted: 5th of June 2017

KEYWORDS

Molybdenum
Methylene blue
Serratia
bioremediation
heavy metals

ABSTRACT

Bioremediation of toxic compound in polluted environment is currently considered as the most economical and ecofriendly approach, particularly at a lower concentration of the toxicant, where other physicochemical techniques are ineffective. In this work, molybdenum-reducing bacterium with the capacity to decolorize various azo and triphenyl methane dyes independent of molybdenum reduction was isolated from contaminated soil. The bacterium reduces molybdate to Mo-blue optimally at pH between 5.8 and 6.5, temperature, between 34 and 37 ^{o}C, molybdate concentration between 10 and 25 mM and phosphate concentration, 5.0 mM. Glucose was the best electron donor supporting molybdate reduction followed by sucrose, maltose, trehalose, d-mannose, glycerol, d-mannitol, d-sorbitol, myo-inositol, d-adonitol and salicin in descending order. The absorption spectrum of Mo-blue produced was similar to other previous Mo-reducing bacteria, and closely resembles a reduced phosphomolybdate. About 78.1, 63.4, 45.5 and 17.8% of the molybdenum reduction in this bacterium was inhibited by 2 ppm mercury (ii), silver (i), copper (ii) and chromium (vi), respectively. The biochemical analysis resulted in a tentative identification of the bacterium as *Serratia marcescens* strain KIK-1. The ability of this bacterium to detoxify molybdenum and decolorize azo dye makes this bacterium an important tool for bioremediation.

INTRODUCTION

Perhaps, the most feasible and economical technique for removal of toxic compounds is bioremediation. This technique surpasses other physicochemical methods in recent time for being effective particularly at a lower concentration of the toxicant. Molybdenum is an essential heavy metal needed by most organisms in a trace amount, however, toxic to a variety of organisms at elevated levels [1]. It has a variety of industrial applications such as alloying agent, a component of corrosion resistant steel, the lubricant in the form of molybdenum disulphide and as automobile engine anti-freeze component. The widespread use of molybdenum in the industry has led to a number of water pollution cases around the world. For example in the Tokyo Bay, Tyrol in Austria, the Black Sea, where levels of molybdenum reached hundreds of ppm [2,3]. Additionally, sewage sludge pollution has been recognized as a significant pollutant terrestrially, which poses a health hazard in the 1970s [4]. At low levels (several parts per million), molybdenum has been reported to inhibit spermatogenesis and arrest embryogenesis in some organisms such as catfish and mice [5–8].

Moreover, molybdenum is very toxic to ruminants, with cows being the most affected [9,10]. Apart from heavy metals, azo dyes as an organic pollutant are often present as co-contaminants in the polluted areas. Nearly, one million tons of basic and diazo direct dyes are produced yearly. Methylene blue (**Fig. 1**) as the basic and cationic dye has found widespread usage as indicator and colorant; its pollution has been documented in water bodies and soils causing serious harm to aquatic life [11][12]. Some of its toxic effects include its potential to cause mutation and cancer in humans and affect the photosynthetic ability of plants [13][14]. Other disease

conditions such as acute renal failure, hyperbilirubinemia and hemolytic anemia have been associated to methylene blue [15]. A study on human cell lines, SK-N-MC human neuroblastoma and U-373 MG human astrocytoma cells have indicated the cytotoxic effects of methylene blue even at micromolar levels [16], as such its urgent removal from the environment is of great importance.

Fig. 1. The structure of methylene blue (Zollinger).

Some microorganisms are able to simultaneously degrade a variety of xenobiotics and detoxify heavy metals [17–19]. The astonishing and versatile capability of these microbes needed in polluted sites where several mixed contaminants are the norm [2]. Previously, heavy metals reduction coupled with azo dye de-colorization has been reported [19].

In this work, the ability of a novel molybdenum-reducing bacterium isolated from contaminated soil to decolorize several azo dyes was ascertained. Static growth condition was purposely used in a microplate environment where oxygen concentration (0~10% environmental oxygen, EO) is lower than under aerobic conditions (~20% environmental oxygen, EO). Since most bioremediation conditions would be performed in aquatic bodies or soils where the EO level is below ~20% EO and nitrate as electron acceptors would thus be used. Here, a novel molybdenum-reducing bacterium with the capacity to decolorize the azo dye methylene blue isolated from contaminated soil is reported. The characteristic nature of this bacterium would make it suitable for future remediation works involving both the heavy metal molybdenum and dye as an organic contaminant.

MATERIALS AND METHODS

Isolation of a molybdenum-reducing bacterium

Soil samples were collected (5 cm deep above topsoil) from contaminated land in Kano, Kano State, Nigeria, in 2014. The soil sample (1 g) was suspended in sterile tap water, 100 µL aliquot of the soil suspension was spread onto agar of low phosphate media (pH 7.0) and incubated at room temperature for 48 hours. The composition of the low phosphate media (LPM) were as follows: Na_2HPO_4 (0.071% or 5 mM), $Na_2MoO_4.2H_2O$ (0.242 % or 10 mM) glucose (1%), $(NH_4)_2.SO_4$ (0.3%), NaCl (0.5%), $MgSO_4.7H_2O$ (0.05%) and yeast extract (0.5%) [20].

The formation of blue colonies indicates molybdate reduction by a molybdenum-reducing bacterium. Colony forming the most intense blue colour was isolated and re-streaked on LPM to obtain a pure culture. In a liquid media (pH 7.0), molybdenum reduction was performed in the above media (100 mL) in a 250 mL shake flask culture on an orbital shaker (120 rpm) at room temperature for 48 hours. Following the required incubation, an aliquot (1.0 mL) of the molybdenum blue (Mo-blue) formed was taken and centrifuged at 10,000 × g for 10 minutes at room temperature. Scanning the spectra of the Mo-blue intensity was carried out from 400 to 900 nm using Shimadzu UV-spectrophotometer (Shimadzu 1201), with LMP was utilized as the baseline correction.

Morphological and biochemical characterization of the Mo-reducing bacterium

The bacterium was phenotypically and biochemically characterized using standard methods such as colony shape, color and size on the nutrient agar plate, motility, gram staining, catalase production and oxidase (24 h), indole production; acetate, malonate and citrate utilization (Simmons); urea, gelatin and esculin hydrolysis, ONPG (beta-galactosidase), ornithine decarboxylase (ODC), arginine dihydrolase (ADH), lysine decarboxylase (LDC), nitrates reduction, methyl red, Voges-Proskauer (VP), hydrogen sulfide (H_2S), tartrate (Jordans), deoxyribonuclease, lipase (corn oil), phenylalanine deaminase, acids production from various sugars and gas production from glucose were performed according to the Bergey's manual of determinative bacteriology [21]. Results interpretation was done via the ABIS online system [22].

Preparation of resting cells for molybdenum reduction characterization

Characterization works on molybdate reduction to Mo-blue such as the effects of temperature, pH, electron donor source, phosphate and molybdate concentrations were statically performed using resting cells in a microplate as developed previously [23]. Bacterial cells were cultured overnight in 1 L high phosphate media (HPM) on an orbital shaker (150 rpm) at room temperature, with phosphate concentration being the only difference between the LPM and HPM, with the later containing 100 mM phosphate. The cultured cells were harvested by centrifugation at 10,000 × g for 15 minutes, and the pellet was washed several times to remove residual phosphate, then re-suspended in 20 mL of LPM devoid glucose to an approximate absorbance 1.00 at 600 nm.

Phosphate concentration (5 mM) in low phosphate media was optimal for all Mo-reducing bacteria isolated so far. Hence, this concentration was chosen for this work, since higher concentrations were found to strongly inhibit molybdate reduction to Mo-blue [20,24–38]. The prepared LMP (180 µL) was sterically pipetted into each well of a sterile microplate, and 20 µL of sterilized glucose solution was then added each to initiate Mo-blue production. A sterile sealing tape that enables gas exchange was used to seal the plate (Corning® microplate) and the microplate culture was incubated at room temperature. Absorbance at 750 nm was periodically measured using BioRad microtiter plate reader (Richmond, CA model No. 680). The concentration of the Mo-blue produced in the microplate cultured media was measured using the specific extinction coefficient of 11.69 $mM.^{-1}.cm^{-1}$ at 750 nm since the maximum filter wavelength available in the microplate unit is 750 nm [39].

Effect of heavy metals on molybdenum reduction

Heavy metals (10) namely arsenic (v), cadmium (ii), chromium (vi), cobalt (ii), copper (ii), lead (ii), mercury (ii), nickel (ii), silver (i) and zinc (ii), were either prepared from commercial salts or from Atomic Absorption Spectrometry (AAS) standard solutions (MERCK, Germany). The bacterial culture media was supplemented with various concentrations of the heavy metals in the microplate format, and the amount of Mo-blue produced was measured at 750 nm as above.

Screening for bacterial decolorization of dyes

The dyes were procured from Sigma-Aldrich (St. Loius, U.S.A.), the bacterial ability to decolorize various dyes was tested in the microplate format above with 100 mg/L dyes added as final concentration. The list with maximum wavelength in parentheses were as follows: Ponceau S (C.I. 27195) (352 nm), Ponceau 2R (C.I. 16150) (388 nm), Metanil Yellow (C.I. 13065) (414 nm), Tartrazine (C.I. 19140) (427 nm), Orange G (C.I. 16230) (476 nm), Crocein Orange G (C.I. 15970) (482 nm), Orange II sodium salt (C.I. 15510) (483 nm), Methyl Red (C.I. 13020) (493 nm), Congo Red (C.I. 22120) (498 nm), Methyl Orange (C.I. 13025) (505 nm), Safranin O (C.I. 50240) (530 nm), Rhodamine B (C.I. 45170) (554 nm), Cresol Red (C.I. 1733-12-6) (570 nm), Nigrosin (C.I. 50415) (570 nm), Direct Blue 71 (C.I. 34140) (586 nm), Crystal Violet (C.I. 42555) (590 nm), Methylene Blue (C.I. 52015) (590 nm), Evans Blue (C.I. 23860) (594 nm), Remazol Black B (C.I. 20505) (597 nm), Sudan Black B (C.I. 26150) (600 nm), Trypan Blue (C.I. 23850) (607 nm), Naphthol Blue Black (C.I. 20470) (618 nm), Fast Green FCF (C.I. 42053) (620 nm), Fuchsin Basic (C.I. 42510) (625 nm), Toluidine Blue (C.I. 52040) (626 nm) and Methyl Green (C.I. 42590) (635 nm).

The constituents of the growth media (at pH 7) include (w/v): $(NH_4)_2.SO_4$(0.3%), $MgSO_4.7H_2O$ (0.05%), $NaNO_3$ (0.2%), Na_2HPO_4 (0.705% or 50 mM), NaCl (0.5%), glucose (1%), sodium lactate (1%) and yeast extract (0.05%). To prevent color changes due to pH, as did occur in some dyes, the phosphate concentration was increased to 50 mM at pH 7.0. Three standard wavelengths (405, 490 and 595 nm) that cover maximum absorption values for specific dyes were used to monitor the decolorization pattern since these wavelengths are available in the BioRad 680 microplate reader.

Additionally, these pre-set wavelengths were used taken into cognisance the maximum absorption spectra for water-soluble dyes were generally shallow, and a slight difference (±) of 20 nm from the wavelength of maximum absorption does not result in dramatic reduction of the absorbance values. The difference in absorbance values from the initial measurements was thus subtracted from the final measurements after 48 hours of incubation, and the percentage decolorization was thus calculated.

Statistical analysis

Results were expressed as means ± SE. Data analyses were carried out using GraphPad InStat version 3.05 and GraphPad Prism version 3.0 available from www.graphpad.com. Analysis of variance (one-way ANOVA) or student's t-test with Tukey's post hoc test was performed to compare between groups. $P<0.05$ was considered statistically significant.

RESULTS

Identification of molybdenum reducing bacterium

The bacterium is Gram-negative, short rod-shaped, motile and facultative anaerobe. The colonies were cream-white, with smooth, circular and shiny appearance between 1 to 3 mm in diameter. This isolate was identified by comparing the morphological and various biochemical characteristics (**Table 1**) to the Bergey's manual of determinative bacteriology [21] and using the ABIS online software [22]. The software gave about three suggestions to the bacterial identity, with >93% highest homology and 95% accuracy to *Serratia marcescens*. More work, especially on molecular identification (16srRNA gene), is required to identify this species further. However, now the bacterium was identified tentatively as *Serratia marcescens* strain KIK-1.

Table 1. Biochemical tests for *Serratia marcescens* strain KIK-1.

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

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

Previous works indicated that *Serratia* genus is the dominant genus for molybdenum reduction with more than six strains isolated so far. Jan [40] reported the first work on molybdate reduction to Mo-blue using *Serratia* sp., followed by *S. marcescens* strain Dr.Y6 [27], *Serratia* sp. Dr.Y5 [28] and *S. marcescens* DR.Y9 [20]. Similarly, some species of this genus have also been reported to decolorize azo dyes [41–44].

A simple and rapid throughput method involving microplate format was utilized in this research to speed up characterization work and obtain more data than the normal shake-flask approach [23,45]. [25] initiated the use of resting cells under static conditions to characterize molybdenum reduction in the bacterium. Resting cells have been used to study heavy metals reduction as in chromate [46], selenate [47], vanadate [48] reductions and also biodegradation of xenobiotics like amides [49], diesel [50], pentachlorophenol [51], phenol [52] and SDS [53].

Molybdenum blue absorption spectrum

The Mo-blue produced by *Serratia marcescens* strain KIK-1 exhibited a characteristic peak between 860 and 870 nm (near infra-red) and a shoulder at approximately 700 nm (**Fig. 2**). The complex structure of the Mo-blue makes it difficult to ascertain its identity as it has many lacunary species [26]. As a reduced product of two molybdenum complexes- isopolymolybdate and heteropolymolybdate, the formation of isopoly Mo-blue from molybdenum is not biologically feasible as this conversion requires strong reducing agents and should be under acidic conditions.

Perhaps, heteropoly Mo-blue formation by enzymatic reduction and biologically-based reducing agents like ascorbic acids is more plausible as seen in ascorbate-reduce phosphate

determination method [54]. While working on *E. coli* K12, [24] suggested that the Mo-blue produced is a reduced form of phosphomolybdate, though did not provide a plausible mechanism.

We hypothesize that in a media containing phosphate and molybdate, microbial molybdate reduction must proceed via phosphomolybdate intermediate, which is formed as a result of pH decrease that occurs during bacterial growth, signifying that the reduction process requires both biological and chemical processes. The fact that the absorption spectrum of the Mo-blue produced by this bacterium resembles the phosphate determination method with a shoulder around 700 to 720 nm and absorption peak around 880 to 890 nm [54] clearly provides evidence for the hypothesis. It was reported that Mo-blue spectra from all other isolated bacteria to follow this trend [26]. Furthermore, due to the complex structure phosphomolybdate species, the exact identification of the compound must be conducted using NMR and ESR [55].

However, scanning the spectroscopic profile of heteropolymolybdate species is a less cumbersome and accepted method for spectrophotometric characterization [56–59]. Although, the wavelength of maximum absorption for Mo-blue is 865 nm, measurement at 750 nm is enough for routine monitoring of Mo-blue production, though with approximately 30% lower intensity, but sufficient enough for routine monitoring of Mo-blue production as this intensity is much higher than cellular absorption at 600-620 nm [23]. Previously, Mo-blue production monitoring uses several wavelengths such as 710 nm [25] and 820 nm [24].

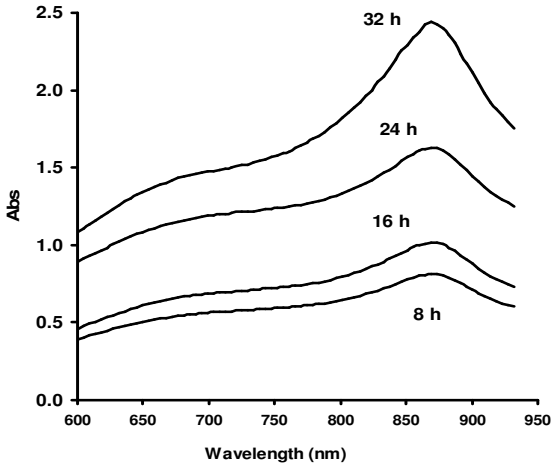

Fig. 2. Absorption spectra scanning for Mo-blue produced by *Serratia marcescens* strain KIK-1 at various time intervals.

Effect of pH and temperature on molybdate reduction

Strain KIK-1 was incubated at various pH (5.5 to 8.0) using Tris.Cl and Bis-Tris buffers (20 mM). ANOVA analysis showed pH between 5.8 and 6.5 as the optimum pH supporting molybdate reduction in this bacterium (**Fig. 3**). A wide range of temperature (20 to 60 °C) was used to study the effect of molybdate reduction in this strain. Temperature between 34 to 37 °C was found to be optimum (**Fig. 4**) with no significant difference ($p>0.05$) among the values measured as analyzed by ANOVA. Temperatures higher than 37 °C were strongly inhibitory to Mo-blue production in *Serratia marcescens* strain KIK-1.

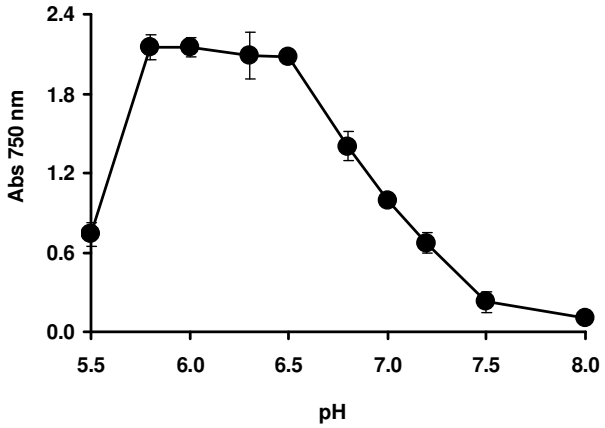

Fig. 3. Effect of pH on Mo-blue production by *Serratia marcescens* strain KIK-1, following 48 hours incubation of the resting cells in a microtiter plate under optimized conditions. Error bars represent mean ± standard deviation (n=3).

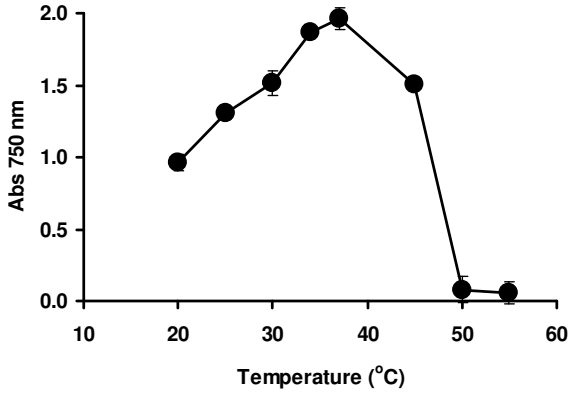

Fig. 4. Effect of temperature on Mo-blue production by *Serratia marcescens* strain KIK-1, following 48 hours incubation of the resting cells in a microtiter plate under optimized conditions. Error bars represent mean ± standard deviation (n=3).

The pH and temperature play an important role in enzyme-mediated molybdate reduction. Both parameters are affected protein structure and folding, hence inhibiting enzyme activity leading to molybdenum reduction. In a tropical country like Malaysia, with average annual temperature ranging from 25 to 35 °, the optimum conditions obtained in this study would be advantageous for bioremediation in this region [27]. Thus, *Serratia marcescens* strain KIK-1 could be a better candidate for bioremediation of molybdenum polluted soil in tropical regions. The vast majority of molybdenum reducers show an optimal temperature between 25 and 37 ºC [20,27,28,30–34,36–38,60] as they were isolated from tropical soils, with only psychrotolerant reducer isolated from Antarctic region that shows temperature between 15 and 20 ºC support optimal molybdate reduction [35]. The optimal pH range shown to support molybdenum reduction by strain KIK-1 reveals its property as a neutrophile, with the ability to grow at pH between 5.5 and 8.0. it was generally observed that during the reduction process in bacteria the pH decreases yielding a slightly acidic with optimal pHs ranging from pH 5.0 to 7.0 [24,25,27–38,60].

It was previously suggested that acidic pH is necessary for the formation and stability of phosphomolybdate intermediate which is being reduced to Mo-blue. Consequently, the optimal

molybdenum reduction occurs by balancing between enzyme activity and substrate stability [61].

Effect of electron donor on molybdate reduction

Amongst the electron donor screened, glucose was the best electron donor source supporting molybdate reduction by strain KIK-1, followed by sucrose, maltose, trehalose, d-mannose, glycerol, d-mannitol, d-sorbitol, myo-inositol, d-adonitol and salicin, whereas, other carbon sources did not support Mo-blue production (**Fig. 5**). It has been reported that most Mo-reducing bacteria such as *E. coli* K12 [62], *Serratia* sp. strain Dr.Y5 [63], *Pseudomonas* sp. strain DRY2 [64], *Pseudomonas* sp. strain DRY1 [35], *Enterobacter* sp. strain Dr.Y13 [29], *Acinetobacter calcoaceticus* strain Dr.Y12 [32], *Bacillus pumilus* strain lbna [65] and *Bacillus* sp. strain A.rzi [66] prefer glucose as the carbon source.

Other molybdenum reducers such as *Enterobacter cloacae* strain 48 [67], *Serratia* sp. strain Dr.Y5 [63], *Serratia marcescens* strain Dr.Y9 [68] and *Serratia marcescens* strain DRY6 [69] utilized sucrose as the best carbon source. However, *Klebsiella oxytoca* strain hkeem prefers fructose [70]. Bacteria metabolizes the carbon source present in the growth medium via pathways such as glycolytic, Kreb's cycle and electron transport chain to produce electron donating substrates, NADH and NADPH, which are responsible for donating an electron to molybdenum reducing-enzyme [60,71].

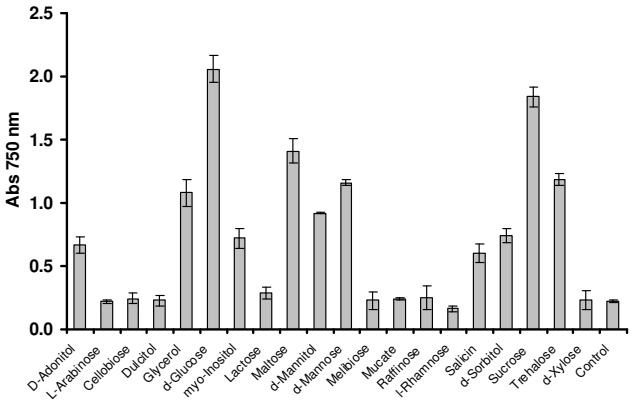

Fig. 5. Effect of various electron donor sources (1% w/v) on Mo-blue production by *Serratia marcescens* strain KIK-1 grown in LMP containing 10 mM molybdate with various electron donors. Bacterial resting cells were incubated for 48 hours in a microtiter plate under optimized conditions. Error bars are mean ± standard deviation (n = 3).

Effect of molybdate and phosphate concentrations on Mo-blue production

Higher phosphate and molybdate concentrations were shown to inhibit Mo-blue production, thus, ascertaining their optimal level is vital [29,32,35,60,64,66,68–70,72]. The optimum phosphate concentration supporting the reduction process occurred at 5 mM, concentrations above which strongly inhibit Mo-blue production (**Fig. 6**). It was earlier suggested that high phosphate concentration inhibit the formation and stability of phosphomolybdate, as this complex requires acidic conditions which is hindered by the strong buffering power of the phosphate buffer that occur at higher phosphate concentration [56,73,74]. Almost all of the molybdenum-reducers isolated so far require phosphate concentration not more than 5 mM for optimal reduction [29,32,35,60,62–67,69,70,72,75,76]. The effect of molybdate concentration on reduction process showed that the newly isolated bacterium was able to tolerate and reduce up to 60 mM molybdate with reduced Mo-blue production intensity. The optimal molybdate reduction occurred between 10 and 25 mM (**Fig. 7**). The lowest optimal molybdate concentration reported was 15 mM in *Pseudomonas* sp strain Dr.Y2 [64], while the highest was 80 mM in *E. coli* K12 [62] and *Klebsiella oxytoca* strain hkeem [70]. Other Mo-reducing bacteria such as *Enterobacter cloacae* 48 [67], *Serratia marcescens* strain Dr.Y6 [69], *Serratia marcescens*. Dr.Y9 [68], *Pseudomonas* sp. strain Dr.Y2 [64], *Serratia* sp. strain Dr.Y5 [63], *Enterobacter* sp. strain Dr.Y13 [29] and *Acinetobacter calcoaceticus* [32] produced maximal Mo-blue at optimal molybdate concentrations 50, 25, 55, 30, 30, 50 and 20 mM, respectively. The highest molybdenum concentration reported as pollutant in the environment was around 2000 ppm or about 20 mM [77]. The potential of this strain to reduce this high concentration to an insoluble Mo-blue would make it efficient to target molybdenum pollution.

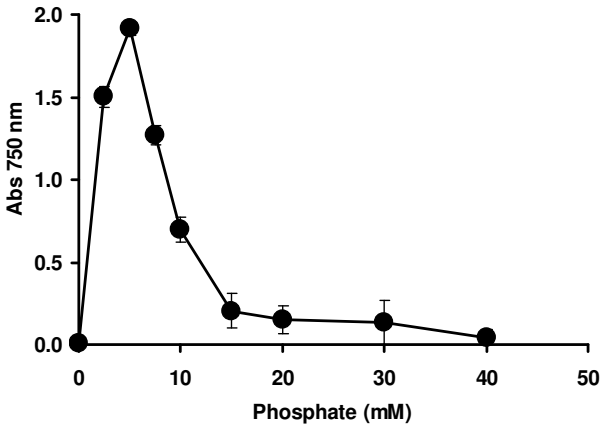

Fig. 6. Effect of phosphate concentration on Mo-blue production by *Serratia marcescens* strain KIK-1, following 48 hours incubation of the resting cells in a microtiter plate under optimized conditions. Error bars are mean ± standard deviation (n = 3).

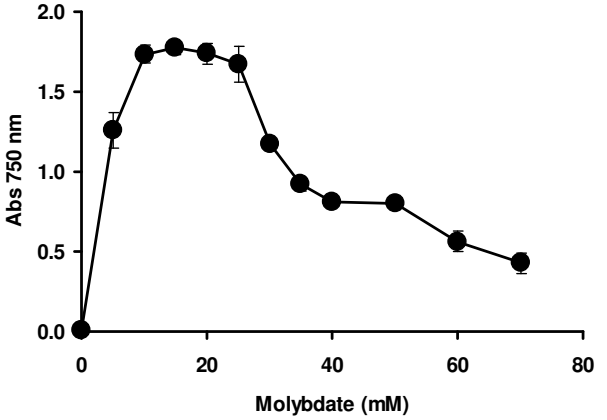

Fig. 7. Effect of molybdate concentration on Mo-blue production by *Serratia marcescens* strain KIK-1, following 48 hours incubation of the resting cells in a microtiter plate under optimized conditions. Error bars are mean ± standard deviation (n = 3).

Effect of heavy metals

The inhibitory effect of other interacting metal ions presents a major problem for bioremediation. Thus, it is of importance to isolate and screen bacteria with as many metal resistance capabilities. Chromium (vi), copper (ii), mercury (ii) and silver (i) at 2 ppm inhibited molybdenum reduction in strain KIK-1 by 17.8, 45.5, 78.1 and 63.4%, respectively (**Fig. 8**). Perhaps, almost all molybdenum reducers are inhibited by a varying

degree of toxic heavy metals as summarized in **Table 2**. Heavy metals such as cadmium, copper, mercury and silver often target the sulfhydryl group of enzymes [78]. Mercury is a physiological inhibitor to molybdate reduction as previously described [79], while chromate is to know to inhibit certain enzymes like glucose oxidase [80] and enzymes involve in nitrogen metabolism in plants [81]. Heavy metals binding inactivated metal-reducing capacity of the enzyme(s) responsible for the reduction.

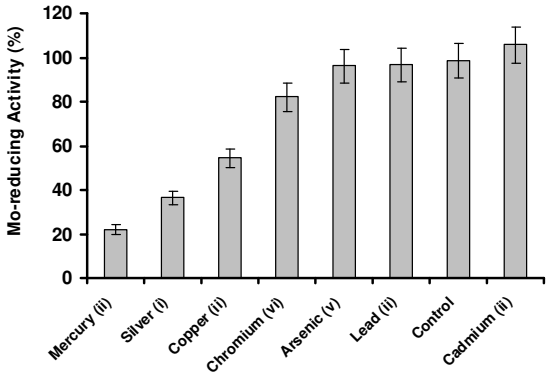

Fig. 8. Effect of metals on Mo-blue production by *Serratia marcescens* strain KIK-1, following 48 hours incubation of the resting cells in a microtiter plate under optimized conditions. Error bars are mean ± standard deviation (n = 3).

Table 2. Inhibition of Mo-reducing bacteria by heavy metals.

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+}	[65]
Bacillus sp. strain A.rzi	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, Hg^{2+}, Co^{2+},Zn^{2+}	[66]
Serratia sp. strain Dr.Y8	Cr, Cu, Ag, Hg	[72]
S. marcescens strain Dr.Y9	Cr^{6+}, Cu^{2+}, Ag^{+}, Hg^{2+}	[68]
Serratia sp. strain Dr.Y5	n.a.	[63]
Pseudomonas sp. strain DRY2	Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	[64]
Pseudomonas sp. strain DRY1	Cd^{2+}, Cr^{6+}, Cu^{2+}, Ag^{+}, Pb^{2+}, Hg^{2+}	[35]
Enterobacter sp. strain Dr.Y13	Cr^{6+}, Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}	[29]
Acinetobacter calcoaceticus strain Dr.Y12	Cd^{2+}, Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	[32]
Serratia marcescens strain DRY6	Cr^{6+}, Cu^{2+}, Hg^{2+}*	[69]
Enterobacter cloacae strain 48	Cr^{6+}, Cu^{2+}	[67]
Escherichia coli K12	Cr^{6+}	[62]
Klebsiella oxytoca strain hkeem	Cu^{2+}, Ag^{+}, Hg^{2+}	[70]

Azo dye-decolorizing ability of the molybdenum-reducing bacterium

This molybdenum-reducing bacterium was screened for its dye decolorizing potential. Strain KIK-1 was able to decolorize methylene blue dye (**Fig. 9**). Among other bacterial species previously reported to degrade this dye which include *Pandoraea norimbergensis*, *Pseudomonas* sp and *Bacillus* sp [82], *Arthrobacter globiformis* [83], *Stenotrophomonas maltophilia* [84].

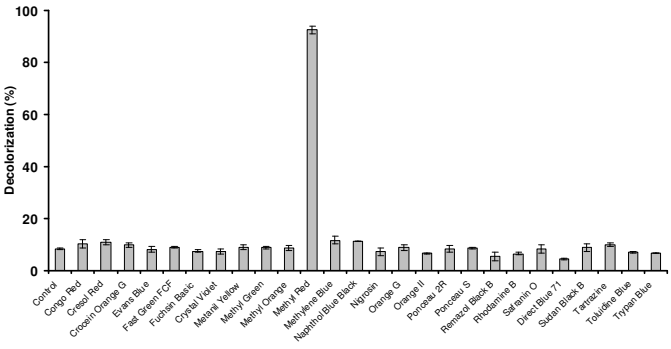

Fig. 9. Dye decolorization by *Serratia marcescens* strain KIK-1, following 48 hours incubation of resting cells in a microtiter plate under optimized conditions. Error bars are mean ± standard deviation (n = 3).

CONCLUSION

This is the first report on a molybdenum-reducing bacterium with methylene blue dye decolorize capacity. The bacterium reduces molybdate to Mo-blue optimally at pH between 5.8 and 6.5, temperature between 34 and 37 ^{o}C, 5 mM phosphate and between 10 and 25 mM molybdate. Glucose was the best electron donor source supporting Mo-blue production followed by sucrose, maltose, trehalose, d-mannose, glycerol, d-mannitol, d-sorbitol, myo-inositol, d-adonitol and salicin, respectively. Molybdenum reduction in this bacterium was inhibited by chromium (vi), copper (ii), mercury (ii) and silver (i). The ability of this bacterium to detoxify molybdenum and decolorize azo dye makes this bacterium an important tool for bioremediation. Research is ongoing to purify molybdenum-reducing enzyme and characterize the decolorization properties of this bacterium.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. AhmadPanahi H, Hosseinzadeh M, Adinehlo H, Moniri E, Manoochehri M. Removal of molybdenum from environmental sample by adsorption using modified aniline-formaldehyde with salicylic acid. World Appl Sci J. 2014;30(12):1892–8.
2. Davis GK. Molybdenum. In: Merian E, editor. Metals and their Compounds in the Environment, Occurrence, Analysis and Biological Relevance. New York: VCH Weinheim; 1991. p. 1089–1100.
3. Neunhäuserer C, Berreck M, Insam H. Remediation of soils contaminated with molybdenum using soil amendments and phytoremediation. Water Air Soil Pollut. 2001;128(1–2):85–96.
4. Lahann RW. Molybdenum hazard in land disposal of sewage sludge. 1976;6(1):3–8.
5. Bi C-M, Zhang Y-L, Liu F-J, Zhou T-Z, Yang Z-J, Gao S-Y, et al. The effect of molybdenum on the in vitro development of mouse preimplantation embryos. Syst Biol Reprod Med. 2013;59(2):69–73.
6. Meeker JD, Rossano MG, Protas B, Diamond MP, Puscheck E, Daly D, et al. Cadmium, lead, and other metals in relation to semen quality: Human evidence for molybdenum as a male reproductive toxicant. Environ Health Perspect. 2008;116(11):1473–9.

7. Zhai X, Zhang Y, Qi Q, Bai Y, Chen X, Jin L, et al. Effects of molybdenum on sperm quality and testis oxidative stress. Syst Biol Reprod Med. 2013 Oct;59:1–5.
8. Zhang Y-L, Liu F-J, Chen X-L, Zhang Z-Q, Shu R-Z, Yu X-L, et al. Dual effects of molybdenum on mouse oocyte quality and ovarian oxidative stress. Syst Biol Reprod Med. 2013;59(6):312–8.
9. Underwood EJ. Environmental sources of heavy metals and their toxicity to man and animals. 1979;11(4–5):33–45.
10. Kincaid RL. Toxicity of ammonium molybdate added to drinking water of calves. J Dairy Sci. 1980;63(4):608–10.
11. Syed MA, Sim HK, Khalid A, Shukor MY. A simple method to screen for azo-dye-degrading bacteria. J Environ Biol. 2009;30(1):89–92.
12. Chung K-T, Stevens Jr. SE. Degradation of azo dyes by environmental microorganisms and helminths. Environ Toxicol Chem. 1993;12(11):2121–32.
13. Aksu Z, Tezer S. Equilibrium and kinetic modelling of biosorption of Remazol black B by *Rhizopus arrhizus* in a batch system: Effect of temperature. Process Biochem. 2000;36(5):431–9.
14. Lucas MS, Amaral C, Sampaio A, Peres JA, Dias AA. Biodegradation of the diazo dye Reactive Black 5 by a wild isolate of Candida oleophila. Enzyme Microb Technol. 2006;39(1):51–5.
15. Albert M, Lessin MS, Gilchrist BF. Methylene blue: Dangerous dye for neonates. J Pediatr Surg. 2003;38(8):1244–5.
16. Lee YS, Wurster RD. Methylene blue induces cytotoxicity in human brain tumor cells. Cancer Lett. 1995;88(2):141–5.
17. Anu M, Gnana TVS, Reshma JK. Simultaneous phenol degradation and chromium (VI) reduction by bacterial isolates. Res J Biotechnol. 2010;5(1):46–9.
18. Bhattacharya A, Gupta A, Kaur A, Malik D. Efficacy of *Acinetobacter* sp. B9 for simultaneous removal of phenol and hexavalent chromium from co-contaminated system. Appl Microbiol Biotechnol. 2014;98(23):9829–41.
19. Chaudhari AU, Tapase SR, Markad VL, Kodam KM. Simultaneous decolorization of reactive Orange M2R dye and reduction of chromate by *Lysinibacillus* sp. KMK-A. J Hazard Mater. 2013;262:580–8.
20. Yunus SM, Hamim HM, Anas OM, Aripin SN, Arif SM. Mo (VI) reduction to molybdenum blue by *Serratia marcescens* strain Dr. Y9. Pol J Microbiol. 2009;58(2):141–7.
21. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. No Title. Bergeys Man Determinative Bacteriol. 1994;
22. Costin S, Ionut S. ABIS online - bacterial identification software, http://www.tgw1916.net/bacteria_logare.html, database version: *Bacillus* 022012-2.10, accessed on Mar 2015. 2015.
23. Shukor MS, Shukor MY. A microplate format for characterizing the growth of molybdenum-reducing bacteria. J Environ Microbiol Toxicol. 2014;2(2):1–3.
24. Campbell AM, Del Campillo-Campbell A, Villaret DB. Molybdate reduction by Escherichia coli K-12 and its chl mutants. Proc Natl Acad Sci U S A. 1985;82(1):227–31.
25. Ghani B, Takai M, Hisham NZ, Kishimoto N, Ismail AKM, Tano T, et al. Isolation and characterization of a Mo6+-reducing bacterium. 1993;59(4):1176–80.
26. Shukor Y, Adam H, Ithnin K, Yunus I, Shamaan NA, Syed A. Molybdate reduction to molybdenum blue in microbe proceeds via phosphomolybdate intermediate. J Biol Sci. 2007;7(8):1448–52.
27. Shukor MY, Habib SHM, Rahman MFA, Jirangon H, Abdullah MPA, Shamaan NA, et al. Hexavalent molybdenum reduction to molybdenum blue by S. Marcescens strain Dr. Y6. Appl Biochem Biotechnol. 2008;149(1):33–43.
28. Rahman MFA, Shukor MY, Suhaili Z, Mustafa S, Shamaan NA, Syed MA. Reduction of Mo(VI) by the bacterium Serratia sp. strain DRY5. J Environ Biol. 2009;30(1):65–72.
29. Shukor MY, Rahman MF, Shamaan NA, Syed MS. Reduction of molybdate to molybdenum blue by Enterobacter sp. strain Dr.Y13. J Basic Microbiol. 2009;49(SUPPL. 1):43–54.
30. Shukor MY, Rahman MF, Suhaili Z, Shamaan NA, Syed MA. Bacterial reduction of hexavalent molybdenum to molybdenum blue. World J Microbiol Biotechnol. 2009;25(7):1225–34.
31. Shukor MY, Ahmad SA, Nadzir MMM, Abdullah MP, Shamaan NA, Syed MA. Molybdate reduction by *Pseudomonas* sp. strain DRY2. J Appl Microbiol. 2010;108(6):2050–8.
32. Shukor MY, Rahman MF, Suhaili Z, Shamaan NA, Syed MA. Hexavalent molybdenum reduction to Mo-blue by *Acinetobacter calcoaceticus*. Folia Microbiol (Praha). 2010;55(2):137–43.
33. Lim HK, Syed MA, Shukor MY. Reduction of molybdate to molybdenum blue by *Klebsiella* sp. strain hkeem. J Basic Microbiol. 2012;52(3):296–305.
34. Abo-Shakeer LKA, Ahmad SA, Shukor MY, Shamaan NA, Syed MA. Isolation and characterization of a molybdenum-reducing *Bacillus pumilus* strain lbna. J Environ Microbiol Toxicol. 2013;1(1):9–14.
35. Ahmad SA, Shukor MY, Shamaan NA, Mac Cormack WP, Syed MA. Molybdate reduction to molybdenum blue by an antarctic bacterium. BioMed Res Int. 2013;(December):1–10.
36. Halmi MIE, Zuhainis SW, Yusof MT, Shaharuddin NA, Helmi W, Shukor Y, et al. Hexavalent molybdenum reduction to Mo-blue by a Sodium-Dodecyl-Sulfate-degrading *Klebsiella oxytoca* strain DRY14. BioMed Res Int. 2013;2013:e384541.
37. Othman AR, Bakar NA, Halmi MIE, Johari WLW, Ahmad SA, Jirangon H, et al. Kinetics of molybdenum reduction to molybdenum blue by Bacillus sp. strain A.rzi. BioMed Res Int. 2013;2013.
38. Khan A, Halmi MIE, Shukor MY. Isolation of Mo-reducing bacterium in soils from Pakistan. J Environ Microbiol Toxicol. 2014;2(1):38–41.
39. Shukor MY, Lee CH, Omar I, Karim MIA, Syed MA, Shamaan NA. Isolation and characterization of a molybdenum-reducing enzyme in Enterobacter cloacae strain 48. Pertanika J Sci Technol. 2003;11(2):261–72.
40. Jan A. 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. 1939;46:336–9.
41. Liang T-W, Chen S-Y, Chen Y-C, Chen C-H, Yen Y-H, Wang S-L. Enhancement of prodigiosin production by Serratia marcescens TKU011 and its insecticidal activity relative to food colorants. J Food Sci. 2013;78(11):M1743–51.
42. Mahmood S, Khalid A, Mahmood T, Arshad M, Ahmad R. Potential of newly isolated bacterial strains for simultaneous removal of hexavalent chromium and reactive black-5 azo dye from tannery effluent. J Chem Technol Biotechnol. 2013;88(8):1506–13.
43. Raj DS, Prabha RJ, Leena R. Analysis of bacterial degradation of azo dye congo red using HPLC. J Ind Pollut Control. 2012;28(1):57–62.
44. Verma P, Madamwar D. Decolourization of synthetic dyes by a newly isolated strain of *Serratia marcescens*. World J Microbiol Biotechnol. 2003;19(6):615–8.
45. Iyamu EW, Asakura T, Woods GM. A colorimetric microplate assay method for high-throughput analysis of arginase activity in vitro. Anal Biochem. 2008;383(2):332–4.
46. Llovera S, Bonet R, Simon-Pujol MD, Congregado F. Chromate reduction by resting cells of *Agrobacterium radiobacter* EPS-916. Appl Environ Microbiol. 1993;59(10):3516–8.
47. Losi ME, Frankenberger Jr. WT. Reduction of selenium oxyanions by *Enterobacter cloacae* strain SLD1a-1: Reduction of selenate to selenite. Environ Toxicol Chem. 1997;16(9):1851–8.
48. Carpentier W, Smet LD, Beeumen JV, Brigé A. Respiration and growth of *Shewanella oneidensis* MR-1 using vanadate as the sole electron acceptor. J Bacteriol. 2005;187(10):3293–301.
49. Raj J, Prasad S, Sharma NN, Bhalla TC. Bioconversion of acrylonitrile to acrylamide using polyacrylamide entrapped cells of *Rhodococcus rhodochrous* PA-34. Folia Microbiol (Praha). 2010;55(5):442–6.
50. Auffret MD, Yergeau E, Labbé D, Fayolle-Guichard F, Greer CW. Importance of Rhodococcus strains in a bacterial consortium degrading a mixture of hydrocarbons, gasoline, and diesel oil additives revealed by metatranscriptomic analysis. 2014.
51. Steiert JG, Pignatello JJ, Crawford RL. Degradation of chlorinated phenols by a pentachlorophenol-degrading bacterium. Appl Environ Microbiol. 1987;53(5):907–10.
52. Sedighi M, Vahabzadeh F. Kinetic Modeling of cometabolic degradation of ethanethiol and phenol by *Ralstonia eutropha*. Biotechnol Bioprocess Eng. 2014;19(2):239–49.

53. Chaturvedi V, Kumar A. Diversity of culturable sodium dodecyl sulfate (SDS) degrading bacteria isolated from detergent contaminated ponds situated in Varanasi city, India. Int Biodeterior Biodegrad. 2011;65(7):961–71.
54. Hori T, Sugiyama M, Himeno S. Direct spectrophotometric determination of sulphate ion based on the formation of a blue molybdosulphate complex. The Analyst. 1988;113(11):1639–42.
55. Chae HK, Klemperer WG, Marquart TA. High-nuclearity oxomolybdenum(V) complexes. Coord Chem Rev. 1993;128(1–2):209–24.
56. Glenn JL, Crane FL. studies on metalloflavoproteins v. the action of silicomolybdate in the reduction of cytochrome c by aldehyde oxidase. Biochim Biophys ACTA. 1956;22:111–5.
57. Sims RPA. Formation of heteropoly blue by some reduction procedures used in the micro-determination of phosphorus. 1961;86(1026):584–90.
58. Kazansky LP, Fedotov MA. 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. 1980;(14):644–6.
59. Yoshimura K, Ishii M, Tarutani T. Microdetermination of phosphate in water by gel-phase colorimetry with molybdenum blue. Anal Chem. 1986;58(3):591–4.
60. Shukor MY, Halmi MIE, Rahman MFA, Shamaan NA, Syed MA. Molybdenum reduction to molybdenum blue in Serratia sp. strain DRY5 is catalyzed by a novel molybdenum-reducing enzyme. BioMed Res Int. 2014;2014:1–8.
61. Shukor Y, Adam H, Ithnin K, Yunus I, Shamaan NA, Syed A. Molybdate reduction to molybdenum blue in microbe proceeds via phosphomolybdate intermediate. J Biol Sci. 2007;7(8):1448–52.
62. Campbell AM, Del Campillo-Campbell A, Villaret DB. Molybdate reduction by Escherichia coli K-12 and its chl mutants. Proc Natl Acad Sci U S A. 1985;82(1):227–31.
63. Rahman MFA, Shukor MY, Suhaili Z, Mustafa S, Shamaan NA, Syed MA. Reduction of Mo(VI) by the bacterium Serratia sp. strain DRY5. J Environ Biol. 2009;30(1):65–72.
64. Shukor MY, Ahmad SA, Nadzir MMM, Abdullah MP, Shamaan NA, Syed MA. Molybdate reduction by Pseudomonas sp. strain DRY2. J Appl Microbiol. 2010;108(6):2050–8.
65. Abo-Shakeer LKA, Ahmad SA, Shukor MY, Shamaan NA, Syed MA. Isolation and characterization of a molybdenum-reducing Bacillus pumilus strain lbna. J Environ Microbiol Toxicol. 2013;1(1):9–14.
66. Othman AR, Bakar NA, Halmi MIE, Johari WLW, Ahmad SA, Jirangon H, et al. Kinetics of molybdenum reduction to molybdenum blue by Bacillus sp. strain A.rzi. BioMed Res Int. 2013;2013:1–9.
67. Ghani B, Takai M, Hisham NZ, Kishimoto N, Ismail AKM, Tano T, et al. Isolation and characterization of a Mo6+-reducing bacterium. 1993;59(4):1176–80.
68. Yunus SM, Hamim HM, Anas OM, Aripin SN, Arif SM. Mo (VI) reduction to molybdenum blue by Serratia marcescens strain Dr. Y9. Pol J Microbiol. 2009;58(2):141–7.
69. Shukor MY, Habib SHM, Rahman MFA, Jirangon H, Abdullah MPA, Shamaan NA, et al. Hexavalent molybdenum reduction to molybdenum blue by S. Marcescens strain Dr. Y6. Appl Biochem Biotechnol. 2008;149(1):33–43.
70. Lim HK, Syed MA, Shukor MY. Reduction of molybdate to molybdenum blue by Klebsiella sp. strain hkeem. J Basic Microbiol. 2012;52(3):296–305.
71. Shukor MY, Rahman MFA, Shamaan NA, Lee CH, Karim MIA, Syed MA. An improved enzyme assay for molybdenum-reducing activity in bacteria. Appl Biochem Biotechnol. 2008;144(3):293–300.
72. Shukor MY, Rahman MF, Suhaili Z, Shamaan NA, Syed MA. Bacterial reduction of hexavalent molybdenum to molybdenum blue. World J Microbiol Biotechnol. 2009;25(7):1225–34.
73. Sims RPA. Formation of heteropoly blue by some reduction procedures used in the micro-determination of phosphorus. 1961;86(1026):584–90.
74. Shukor MY, Shamaan NA, Syed MA, Lee CH, Karim MIA. 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. 2000;8(2):167–72.
75. Halmi MIE, Zuhainis SW, Yusof MT, Shaharuddin NA, Helmi W, Shukor Y, et al. Hexavalent molybdenum reduction to Mo-blue by a Sodium-Dodecyl-Sulfate-degrading Klebsiella oxytoca strain DRY14. BioMed Res Int. 2013;2013:e384541.
76. Khan A, Halmi MIE, Shukor MY. Isolation of Mo-reducing bacterium in soils from Pakistan. J Environ Microbiol Toxicol. 2014;2(1):38–41.
77. Runnells DD, Kaback DS, Thurman EM, Chappel WR, Peterson KK. Geochemistry and sampling of molybdenum in sediments, soils, and plants in Colorado. Molybdenum Environ. 1976;
78. Sugiura Y, Hirayama Y. Structural and electronic effects on complex formation of copper(II) and nickel(II) with sulfhydryl-containing peptides. Inorg Chem. 1976;15(3):679–82.
79. Shukor MY, Syed MA, Lee CH, Karim MIA, Shamaan NA. A method to distinguish between chemical and enzymatic reduction of molybdenum in Enterobacter cloacae strain 48. Malays J Biochem. 2002;7:71–2.
80. Zeng G-M, Tang L, Shen G-L, Huang G-H, Niu C-G. 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. 2004;84(10):761–74.
81. Sangwan P, Kumar V, Joshi UN. Effect of chromium(VI) toxicity on enzymes of nitrogen metabolism in clusterbean (cyamopsis tetragonoloba L.). Enzyme Res. 2014;2014.
82. Bandounas L, Wierckx NJP, de Winde JH, Ruijssenaars HJ. Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential. BMC Biotechnol. 2011;11.
83. Itoh K. Decolorization and degradation of methylene blue by Arthrobacter globiformis. Bull Environ Contam Toxicol. 2005;75(6):1131–6.
84. Galai S, Limam F, Marzouki MN. A new *Stenotrophomonas maltophilia* strain producing laccase. use in decolorization of synthetics dyes. Appl Biochem Biotechnol. 2009;158(2):416–31.