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Isolation and Characterization of a Molybdenum-reducing and Orange G-decolorizing *Enterobacter* sp. strain Zeid-6 in Soils from Sudan

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ABSTRACT

Chemical toxins and organic contaminants such as hydrocarbons and dyes are major global contaminants with countless tones of those chemicals are created yearly with a significant amount release to the environment. In this work we screen the ability of a molybdenum-reducing bacterium isolated from contaminated soil to decolorize various azo and triphenyl methane dyes independent of molybdenum reduction. The bacterium was able to decolorize the azo dye Orange G. The bacterium reduces molybdate to Mo-blue optimally at pH between 5.5 and 8.0 and temperatures of between 30 and 37 ºC. Other requirements include a phosphate concentration of 5 mM and a molybdate concentration of 20 mM. The absorption spectrum of the Mo-blue produced was similar to previous Mo-reducing bacterium, and closely resembles a reduced phosphomolybdate. Molybdenum reduction was inhibited by copper, mercury, silver and chromium with percentage of inhibition of 59.5, 78.9, 69.2 and 40.1 %, respectively. The resultant molybdenum blue spectrum closely resembles the spectrum of molybdenum blue from the phosphate determination method. Biochemical analysis resulted in a tentative identification of the bacterium as *Enterobacter* sp. strain Zeid-6. The ability of this bacterium to detoxify molybdenum and decolorize azo dye makes this bacterium an important tool for bioremediation.

INTRODUCTION

Pollutants and organic contaminants such as hydrocarbons and dyes are key global toxic contamination with countless tonnes of these chemical substances are manufactured yearly having a significant amount discharge to the environment. Molybdenum is required at trace levels as it is the component of many enzymes as cofactor in the form of molybdopterin [1]. It has many uses in industries and this wide application is a major contribution to soil and water bodies pollution [2,3]. Molybdenum has also been reported from the mining industry, and sites across the world has been found to be contaminated with this metals [4–6]. As new evidence has indicated that molybdenum is toxic to processes such as spermatogenesis and embryogenesis [7–9], its further removal from the environment is warranted.

Azo dyes including Orange G (**Fig.** 1) is part of the largest group of dyes from the existing 10,000 commercial dyestuffs. It has been documented that during industrial processes using these dyes, it is estimated that around 15% of the dye is lost as wastewater in the effluents. These dyes in the wastewater are not easily removed from and pose a hazard to the environment [10]. Orange G is also toxic with several studies on its toxicity in rats and pigs have been carried out. Pigs exposed to the dye showed higher levels of Heinz bodies in the erythrocytes. In addition toxicity signs such as anaemia, reticytosis and splenomegaly was observed [11]. Orange G fed to rats at 5000 ppm resulted in the formation of Heinz bodies in the

erythrocytes with the presence of anaemia, methaemoglobinaemia, reticulocytosis and splenomegaly [12]. The toxicity of this dye, and its reported pollution in the environment means efficient microbial strains for its removal will need to be discovered as biological methods are more efficient and economic than physical and chemical methods.

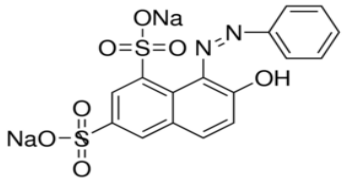

Fig. 1. The structure of Orange G [13].

Some microbes are able to degrade a variety of xenobiotics and detoxify heavy metals at the same time [14,15] and the versatility of these microbes are in great demand in polluted sites where the presence of several contaminants are the norm. Molybdenum reduction coupled with azo dye decolorization have been reported before [16]. This new and novel aspect of molybdenum decolorization is important to be expanded, and more dye decoloring molybdenum reducers should be discovered. Here we report on a novel molybdenum-reducing bacterium with the capacity to decolorize the azo dye Orange G isolated from a contaminated soil. The characteristics of this bacterium would make it suitable for future bioremediation works involving both the heavy metal molybdenum and dye as an organic contaminant.

MATERIALS AND METHODS

Isolation of molybdenum-reducing bacterium

Soil samples were taken (5 cm deep from topsoil) from the grounds of a contaminated land in the city of Juba, South Sudan, Africa in 2012. One gram of soil sample was suspended in sterile tap water. 0.1 mL aliquot of the soil suspension was pipetted and spread onto agar of low phosphate media (pH 7.0), and incubated for 48 hours at room temperature. The composition of the low phosphate media (LPM) were as follows: glucose (1%), $(NH_4)_2.SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), yeast extract (0.5%), NaCl (0.5%), $Na_2MoO_4.2H_2O$ (0.242 % or 10 mM) and Na_2HPO_4 (0.071% or 5 mM) [17]. The formations of blue colonies indicate molybdate reduction by molybdenum-reducing bacteria.

Colony forming the strongest blue intensity was isolated and restreaked on low phosphate media (LPM) to obtain pure culture. Molybdenum reduction in liquid media (at pH 7.0) was carried out in 100 mL of the above media in a 250 mL shake flask culture at room temperature for 48 hours on an orbital shaker set at 120 rpm with the same media above but the phosphate concentration increased to 100 mM. Molybdenum blue (Mo-blue) absorption spectrum was studied by taking out 1.0 mL of the Mo-blue formed from the liquid culture above and then centrifuged at 10,000 x g for 10 minutes at room temperature. Scanning of the supernatant was carried out from 400 to 900 nm using a UV-spectrophotometer (Shimadzu 1201). The low phosphate media was utilized as the baseline correction.

Morphological, physiological and biochemical characterization of the Mo-reducing bacterium

The bacterium was partially identified biochemically and phenotypically using standard methods such as colony shape, gram staining, size and colour on nutrient agar plate, motility, oxidase (24 h) and other biochemical tests according to the Bergey's Manual of Determinative Bacteriology [18]. Interpretation of the results was carried out via the ABIS online system [19].

Preparation of resting cells for molybdenum reduction characterization

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

In the low phosphate media, a concentration of 5 mM phosphate was optimal for all of the Mo-reducing bacteria isolated so far and hence this concentration was used in this work. Higher concentrations were found to be strongly inhibitory to molybdate reduction [17,21–35]. Then 180 µL was sterically pipetted into each well of a sterile microplate. 20 µL of sterile glucose from a stock solution was then added to each well to initiate Mo-blue production. A sterile sealing tape that allows gas exchange (Corning® microplate) was used for sealing the tape.

The microplate was incubated at room temperature. At defined times, absorbance at 750 nm was read in a BioRad (Richmond, CA) Microtiter Plate reader (Model No. 680). The production of Mo-blue from the media in a microplate format was measured using the specific extinction coefficient of 11.69 $mM.^{-1}.cm^{-1}$ at 750 nm as the maximum filter wavelength available for the microplate unit was 750 nm [36].

Effect of heavy metals on molybdenum reduction

Ten heavy metals namely chromium, zinc, mercury, cadmium, lead, nickel, coppe, arsenic, cobalt and silver were tested in this study. These metal ions were either prepared from commercial salts or from prepared standards such as from the Atomic Absorption Spectrometry standard solutions from MERCK. The bacterium was incubated with heavy metals in the microplate format at various concentrations. The amount of Mo-blue production was measured at 750 nm as before.

Screening for bacterial decolorization of dyes

The ability of the bacterium to decolorize dyes was tested using the microplate format above with the dyes added to the final concentration of 100 mg/L. Dyes were sourced from Sigma-Aldrich (St. Loius, U.S.A.) and the list with maximum wavelength in parentheses were as follows: Congo Red (C.I. 22120) (498 nm), Cresol Red (C.I. 1733-12-6) (570 nm), Crocein Orange G (C.I. 15970) (482 nm), Evans Blue (C.I. 23860) (594 nm), Fast Green FCF (C.I. 42053) (620 nm), Fuchsin Basic (C.I. 42510) (625 nm), Crystal Violet (C.I. 42555) (590 nm), Metanil Yellow (C.I. 13065) (414 nm), Methyl Green (C.I. 42590) (635 nm), Methyl Orange (C.I.

13025) (505 nm), Methyl Red (C.I. 13020) (493 nm), Methylene Blue (C.I. 52015) (590 nm), Naphthol Blue Black (C.I. 20470) (618 nm), Nigrosin (C.I. 50415) (570 nm), Orange G (C.I. 16230) (476 nm), Orange II sodium salt (C.I. 15510) (483 nm), Ponceau 2R (C.I. 16150) (388 nm), Ponceau S (C.I. 27195) (352 nm), Remazol Black B (C.I. 20505) (597 nm), Rhodamine B (C.I. 45170) (554 nm), Safranin O (C.I. 50240) (530 nm), Direct Blue 71 (C.I. 34140) (586 nm), Sudan Black B (C.I. 26150) (600 nm), Tartrazine (C.I. 19140) (427 nm), Toluidine Blue (C.I. 52040) (626 nm) and Trypan Blue (C.I. 23850) (607 nm).

The ingredients of the growth media (% w/v) (LPM) were as follows: Glucose (1%), sodium lactate (1%), $(NH_4)_2.SO_4$ (0.3%), $NaNO_3$ (0.2%), $MgSO_4.7H_2O$ (0.05%), yeast extract (0.05%), NaCl (0.5%), Na_2HPO_4 (0.705% or 50 mM). The media was adjusted to pH 7.0. Some of the dyes change in color as the pH changes and the phosphate concentration was increased to 50 mM at pH 7.0 to prevent this.

Decolorization was monitored using three standard wavelengths which were 405, 490 and 595 nm to cover maximum absorption values for specific dyes as these wavelengths were available in the BioRad 680 microplate reader. In addition, the use of these pre set wavelengths takes into account that the maximum absorption spectrums of water soluble dyes were generally shallow and a ± difference of 20 nm from the maximum absorption wavelength does not give dramatic reduction of absorbance values. The difference of absorbance values from the initial measurements were subtracted from the final measurements after an incubation period of 48 hours and a percentage decolorization was calculated.

Statistical analysis

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

RESULTS AND DISCUSSION

Identification of molybdenum reducing bacterium

Molybdenum reduction by bacteria offers an alternative method to the physicochemical method of its removal. Removal can be carried out at less cost and is an environmental friendly approach. Strain Zeid-6 was a short rod-shaped, motile, Gram-negative and facultative anaerobe bacterium. The bacterium was identified by comparing the results of cultural, morphological and various biochemical tests (**Table** 1) to the Bergey's Manual of Determinative Bacteriology and using the ABIS online software [19].

The software gave three suggestions for the bacterial identity with the highest similarity or homology (90%) and accuracy (100%) as *Enterobacter cloacae*. However, more work in the future especially molecular identification technique through comparison of the 16srRNA gene are needed to identify this species further. However, at this juncture the bacterium is tentatively identified as *Enterobacter* sp. strain Zeid-6 in honor of the late Dr. Neni Gusmanizar. Previously, two molybdenum-reducing bacterium from this genus; *Enterobacter cloacae* strain 48 [22] and *Enterobacter* sp. strain Dr.Y13 [26] have been isolated (**Table** 2).

Table 1. Biochemical tests for *Enterobacter* sp. strain Zeid-6.

Motility	+	Acid production from:	
Pigment	–	Alpha-Methyl-D-Glucoside	+
Catalase production (24 h)	+	D-Adonitol	+
Oxidase (24 h)	–	L-Arabinose	+
ONPG (beta-galactosidase)	+	Cellobiose	d
Arginine dihydrolase (ADH)	+	Dulcitol	+
Lysine decarboxylase (LDC)	–	Glycerol	+
Ornithine decarboxylase (ODC)	+	D-Glucose	+
Nitrates reduction	+	myo-Inositol	+
Methyl red	–	Lactose	+
Voges-Proskauer (VP)	+	Maltose	+
Indole production	–	D-Mannitol	+
Hydrogen sulfide (H2S)	–	D-Mannose	+
Acetate utilization	+	Melibiose	+
Malonate utilization	+	Mucate	+
Citrate utilization (Simmons)	+	Raffinose	+
Tartrate (Jordans)	+	L-Rhamnose	+
Esculin hydrolysis	+	Salicin	+
Gelatin hydrolysis	–	D-Sorbitol	+
Urea hydrolysis	+	Sucrose	+
Deoxyribonuclease	–	Trehalose	+
Lipase (corn oil)	–	D-Xylose	+
Phenylalanine deaminase	–		
Growth on KCN medium	+		

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

Table 2. List of molybdenum-reducing bacteria isolated to date.

Bacteria	Optimal C source	Optimal Molybdate (mM)	Heavy metals inhibition	Author
Serratia sp. strain MIE2	sucrose	10	Cu^{2+}, Ag^{+}, Hg^{2+}	[37]
Serratia marcescens strain Neni-1	glucose	10-20	Cu^{2+}, Ag^{+}, Hg^{2+} Cr^{6+}	[16]
Burkholderia sp. strain Neni-11	glucose	15	Hg^{2+}, Ag^{+}, Cr^{6+}	[38]
Klebsiella oxytoca strain Saw-5	glucose	20-30	Cu^{2+}, Ag^{+}, Hg^{2+} Cd^{2+}	[39]
Pseudomonas aeruginosa strain Amr-11	glucose	20-30	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Hg^{2+},	[40]
Klebsiella oxytoca strain Aft-7	glucose	5-20	Cu^{2+}, Ag^{+}, Hg^{2+}	[15]
Bacillus sp. strain A.rzi	glucose	50	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, Hg^{2+}, Co^{2+},Zn^{2+}	[34]
Bacillus pumilus strain lbna	glucose	40	As^{3+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	[31]
Pseudomonas sp. strain DRY1	glucose	30-50	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, Hg^{2+}	[32]
Klebsiella oxytoca strain hkeem	fructose	80	Cu^{2+}, Ag^{+}, Hg^{2+}	[30]
Pseudomonas sp. strain DRY2	glucose	15-20	Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	[28]
Acinetobacter calcoaceticus strain Dr.Y12	glucose	20	Cd^{2+}, Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	[29]
S. marcescens strain Dr.Y9	sucrose	20	Cr^{6+}, Cu^{2+}, Ag^{+}, Hg^{2+}	[17]
Serratia sp. strain Dr.Y8	sucrose	50	Cr, Cu, Ag, Hg	[27]
Enterobacter sp. strain Dr.Y13	glucose	25-50	Cr^{6+}, Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}	[26]
Serratia sp. strain Dr.Y5	glucose	30	n.a.	[25]
Serratia marcescens strain DRY6	sucrose	15-25	Cr^{6+}, Cu^{2+}, Hg^{2+}	[24]
Enterobacter cloacae strain 48	sucrose	20	Cr^{6+}, Cu^{2+}	[22]
Escherichia coli K12	glucose	80	Cr^{6+}	[21]

Molybdenum absorbance spectrum

Analyzing the absorption spectrum of the molybdenum blue product can reveal the identity of the compound. The absorption spectrum of Mo-blue produced by *Enterobacter* sp. strain Zeid-6 exhibited a shoulder at approximately 700 nm and a maximum peak near the infra-red region of between 860 and 870 nm with a median at 865 nm (**Fig.** 2). The identity of the Mo-blue is not easily ascertained as it is complex in structure and has many species [23]. Briefly Mo-blue is a reduced product of two type of molybdenum complexes-isopolymolybdate and heteropolymolybdate. We have shown previously that the entire Mo-blue spectra from other bacteria indicate that they are reduced phosphomolybdate [23]. Although the maximum absorption wavelength for Mo-blue was 865 nm, the wavelength 750 nm was utilized in this work, although it is approximately 30% lower [20]. Other wavelengths have been used in previous monitoring of Mo-blue production that include 710 nm [22] and 820 nm [21].

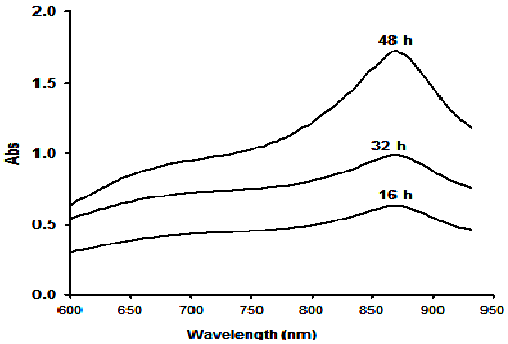

Fig. 2. Scanning absorption spectrum of Mo-blue from *Enterobacter* sp. strain Zeid-6 at different time intervals.

Effect of pH and temperature on molybdate reduction

Enterobacter sp. strain Zeid-6 was incubated at different pH ranging from 5.5 to 8.0 using Bis-Tris and Tris.Cl buffers (20 mM). Analysis by ANOVA showed that the optimum pH for reduction was between 6.0 and 6.5. Inhibition of reduction was dramatic at pH lower than 5 (**Fig.** 3). The effect of temperature (**Fig.** 4) was observed over a wide range of temperature (20 to 60°C) with an optimum temperature ranging from 30°C to 37°C with no significant different ($p>0.05$) among the values measured as analysed using ANOVA. Temperatures lower than 30°C and higher than 37ºC were strongly inhibitory to Mo-blue production from *Enterobacter* sp. strain Zeid-6.

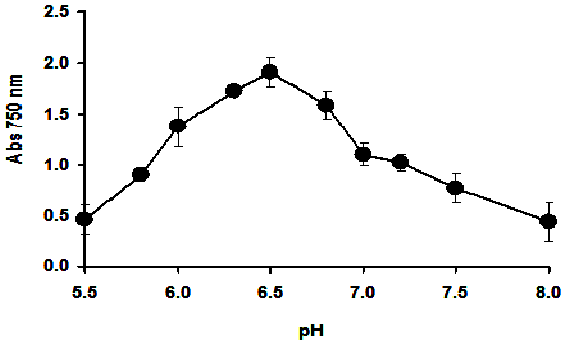

Fig. 3. Effect of pH on molybdenum reduction by *Enterobacter* sp. strain Zeid-6. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 48 hours. Error bars represent mean ± standard deviation (n=3).

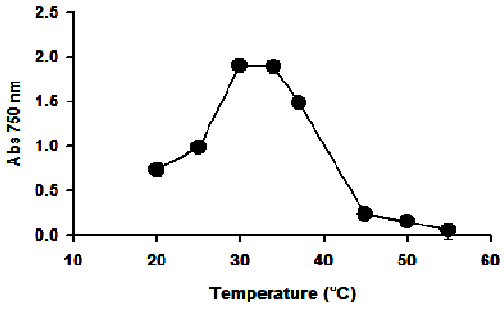

Fig. 4. Effect of temperature on molybdenum reduction by *Enterobacter* sp. strain Zeid-6. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 48 hours. Error bars represent mean ± standard deviation (n=3).

Temperature and pH play important roles in molybdenum reduction, since this process is enzyme mediated. The majority of the reducers including this strain show an optimal temperature of between 25 and 37ºC [17,24,25,27–31,33–35,41] as they are isolated from tropical soils with the only psychrotolerant reducer isolated from Antartica showing an optimal temperature supporting reduction of between 15 and 20ºC [32].

The optimal pH range exhibited by *Enterobacter* sp. strain Zeid-6 for supporting molybdenum reduction reflects the property of the bacterium as a neutrophile. The characteristics neutrophiles are their ability to grow between pH 5.5 and 8.0. An important observation regarding molybdenum reduction in bacteria is the optimal pH reduction is slightly acidic with optimal pHs ranging from pH 5.0 to 7.0 [21,22,24–35,41]. It has been suggested previously that acidic pH plays an important role in the formation and stability of phosphomolybdate before it is being reduced to Mo-blue. Thus, the optimal reduction occurs by balancing between enzyme activity and substrate stability [23].

Effect of electron donor on molybdate reduction

Among the electron donor tested, glucose was the best electron donor for supporting molybdate reduction followed by sucrose, adonitol, mannose, mannitol, myo-inositol, maltose, glycerol, d-sorbitol, salicin, trehalose and xylose (**Fig.** 5). Other carbon sources did not support molybdenum reduction.

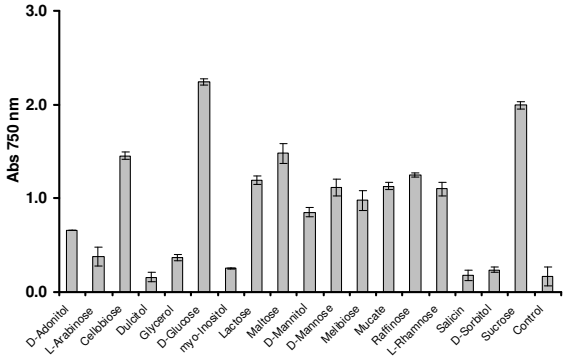

Fig. 5. Effect of different electron donor sources (1% w/v) on molybdenum reduction. *Enterobacter* sp. strain Zeid-6 was grown in low phosphate media containing 10 mM molybdate and various electron donors. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 48 hours. Error bars represent mean ± standard deviation (n = 3).

Previous works by Shukor et al. demonstrated that several of Mo-reducing bacteria such as *Enterobacter cloacae* strain 48 [22], *Serratia* sp. strain Dr.Y5 [25], *S. marcescens* strain Dr.Y9 [17] and *Serratia marcescens* strain DRY6 [24] showed sucrose as the best carbon source. Other molybdenum reducers such as *Escherichia coli* K12 [21], *Serratia* sp. strain Dr.Y5 [25], *Pseudomonas* sp. strain DRY2 [28], *Pseudomonas* sp. strain DRY1 [32], *Enterobacter* sp. strain Dr.Y13 [26], *Acinetobacter calcoaceticus* strain Dr.Y12 [29], *Bacillus pumilus* strain lbna [31] and *Bacillus* sp. strain A.rzi [34] prefer glucose as the carbon source while *Klebsiella oxytoca* strain hkeem prefers fructose [30]. These carbon sources produce the reducing equivalents NADH and NADPH easily through generic metabolic pathways, and both reducing equivalents are known substrates for the Mo-reducing enzyme [41,42]. A less expensive carbon source for instance molasses may be used

over sucrose and glucose in the foreseeable future, especially for bioremediation works considering that molasses can be purchased in great quantity as agricultural waste products particularly sugar cane [43]. Molasses is utilized as a cheap carbon source in the bacterial reduction of hexavalent chromate [44,45] and selenate [46]. The impact of molasses as an alternative carbon source is presently being studied.

Effect of phosphate and molybdate concentrations to molybdate reduction

The determination of phosphate and molybdate concentrations supporting optimal molybdenum reduction is important because both anions have been shown to inhibit Mo-blue production in bacteria [17,24,26–30,32,34,41]. The optimum concentration of phosphate occurred at 5 mM with higher concentrations were strongly inhibitory to reduction (**Fig.** 6). High phosphate was suggested to inhibit phosphomolybdate stability as the complex requires acidic conditions of which the higher the phosphate concentration the stronger buffering power of the phosphate buffer used. In addition, the phosphomolybdate complex itself is unstable in the presence of high phosphate through an unknown mechanism [47–49].

All of the molybdenum-reducing bacterium isolated so far requires phosphate concentration not higher than 5 mM for optimal reduction [21,22,24–35,41]. Studies on the effect of molybdenum concentration on molybdenum reduction showed that the newly isolated bacterium was able to reduce molybdenum as high as 60 mM but with reduced Mo-blue production. The optimal reduction range was between 20 and and 40 mM (**Fig.** 7). Reduction at this high concentration into an insoluble form would allow the strain to reduce high concentration of molybdenum pollution. The lowest optimal concentration of molybdenum reported is 15 mM in *Pseudomonas* sp. strain Dr.Y2 [28], whilst the highest molybdenum required for optimal reduction was 80 mM in *E. coli* K12 [21] and *Klebsiella oxytoca* strain hkeem [30]. Other Mo-reducing bacteria such as EC48 [22], *S. marcescens* strain Dr.Y6 [24], *S. marcescens*. Dr.Y9 [17], *Pseudomonas* sp. strain Dr.Y2 [28], *Serratia* sp. strain Dr.Y5 [25], *Enterobacter* sp. strain Dr.Y13 [26] and *Acinetobacter calcoaceticus* [29] could produce optimal Mo-blue using the optimal molybdate concentrations at 50, 25, 55, 30, 30, 50 and 20 mM, respectively. In fact the highest concentration of molybdenum as a pollutant in the environment is around 2000 ppm or about 20 mM [50].

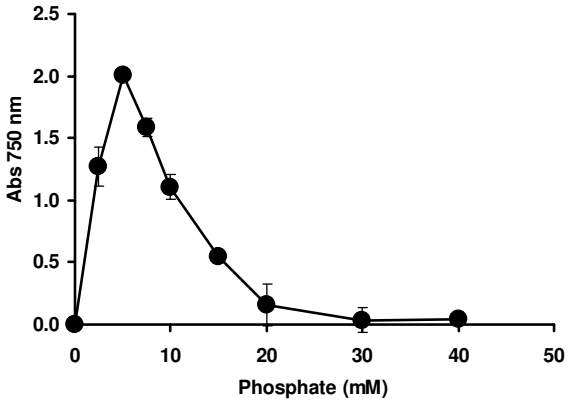

Fig. 6. The effect of phosphate concentration on molybdenum reduction by *Enterobacter* sp. strain Zeid-6. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 48 hours. Error bars represent mean ± standard deviation (n = 3).

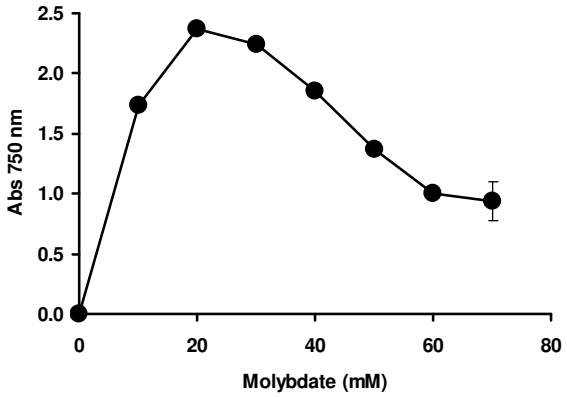

Fig. 7. The effect of molybdate concentration on molybdenum reduction by *Enterobacter* sp. strain Zeid-6. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 48 hours. Error bars represent mean ± standard deviation (n = 3).

Effect of heavy metals

Earlier reports on bacterial molybdenum reduction demonstrate that reduction is strongly inhibited by heavy metals such as chromium, copper and mercury [21,22]. The type of heavy metals that inhibit reduction varies from one reducers to another. Inhibition of molybdenum reduction using several metals at 1 ppm showed that copper, mercury, silver and chromium showed 59.5, 78.9, 69.2 and 40.1 % inhibition to Mo-reducing activity of *Enterobacter* sp. strain Zeid-6 (**Fig.** 8).

The inhibition effects by others metal ions and heavy metals present a major problem for bioremediation. Therefore, it is important to screen and isolate bacteria with as many metal resistance capabilities. As described previously [51], mercury is a physiological inhibitor to molybdate reduction. 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). Heavy metals such as mercury, cadmium, silver and copper usually target sulfhydryl group of enzymes [52]. Chromate is known to inhibit certain enzymes such as glucose oxidase [53] and enzymes of nitrogen metabolism in plants [54]. Binding of heavy metals inactivated metal-reducing capability of the enzyme(s) responsible for the reduction.

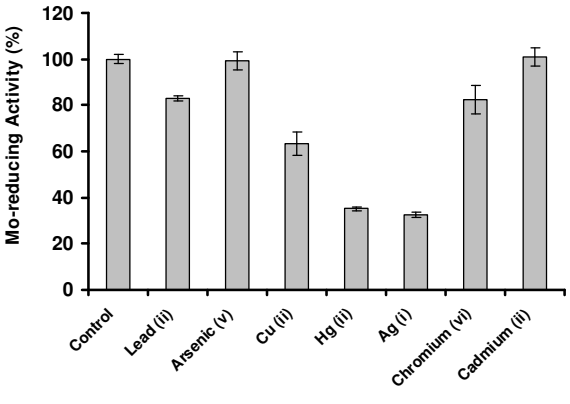

Fig. 8. The effect of metals on Mo-blue production by *Enterobacter* sp. strain Zeid-6. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 48 hours. Error bars represent mean ± standard deviation (n = 3).

Azo dye-decolorizing ability of the molybdenum-reducing bacterium

The Mo-reducing bacterium was then screened for dye decolorizing ability. The bacterium was able to decolorize the dye Orange G (**Fig.** 9). Other azo dyes including Methylene Blue, Crocein Orange G, Direct Blue 71, Congo red, Orange II, Evans Blue, Ponceau S, Remazol Black B or 5, Methyl Orange, Tartrazine, Methyl Red, Naphtol Blue Black and the rest of the other dyes were either poorly degraded or failed to be decolorized at all in comparison to control. Bacterial species that have been reported to be able to degrade this dye include *Kluyvera ascorbata, Bacillus* sp., *Pseudomonas* sp. and *Pasteurella* sp [55], *Bacillus megaterium* [56], *Citrobacter amalonaticus* [57] and *Bacillus fusiformis* [58]. The azo bond (–N=N–) is vulnerable to reductive cleavage (Syed *et al.*, 2009), and this is probably the mechanism of dye decolorization observed in this study.

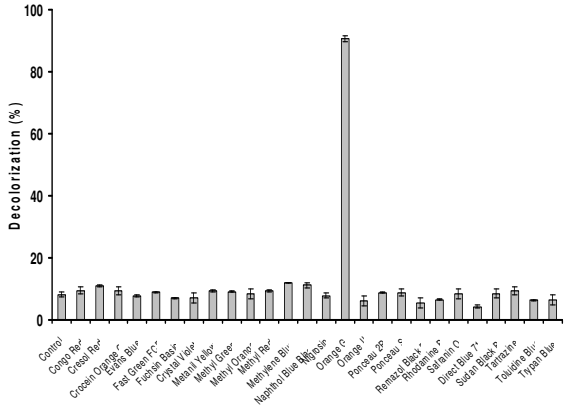

Fig. 9. Dye decolorization ability of *Enterobacter* sp. strain Zeid-6. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 48 hours. Error bars represent mean ± standard deviation (n = 3).

CONCLUSION

A local isolate of Mo-reducing bacterium with the novel ability to decolorize azo dye has been isolated. This is the first report of a molybdenum reducing bacterium with the ability to decolorize the dye Orange G. The bacterium reduces molybdate to Mo-blue optimally at pH between 5.5 and 8.0 and temperatures of between 30 and 37°C. Other requirements include a phosphate concentration of 5 mM and a molybdate concentration of X mM. The absorption spectrum of the Mo-blue produced was similar to previous Mo-reducing bacterium, and closely resembles a reduced phosphomolybdate. Molybdenum reduction was inhibited by copper, mercury, silver and chromium. The ability of this bacterium to detoxify multiple toxicants is a sought after property, and this makes the bacterium an important tool for bioremediation. Currently, work is underway to purify the molybdenum-reducing enzyme from this bacterium and to characterize decolorization studies in more detail.

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