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A Molybdenum-reducing *Bacillus* sp. Strain Zeid 14 in Soils from Sudan that Could Grow on Amides and Acetonitrile

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ABSTRACT

Agricultural and industrial activities contribute most to pollutants found globally, and bioremediation of these pollutants is being intensely sought. We have isolated a molybdenum-reducing bacterium from agricultural soil for bioremediation purposes. The bacterium was grown in low phosphate medium supplemented with molybdate in a microplate format. The molybdenum-reducing bacterium was then further screened for amide-degrading properties. The bacterium was able to use acrylamide as a source of electron donor for reduction, and was able to grow on acrylamide, acetamide and acetonitrile. The growth parameters obtained according to the modified Gompertz model were lag periods of 0.468, 0.979 and 1.53 d and maximum specific growth rates of 1.165, 0.932, 0.842 d^{-1} for acrylamide, acetamide and acetonitrile respectively. Optimal conditions for molybdate reduction included glucose, pH between 6.0 and 6.8, temperature between 25° C and 34° C, and phosphate and molybdate concentrations between 5 and 7.5 mM and 10 and 20 mM, respectively. The Mo-blue exhibited a unique absorption spectrum closely resembling a reduced phosphomolybdate. Mo-blue production was inhibited by the heavy metals copper, mercury, silver, chromium and cadmium. The bacterium was identified as *Bacillus* sp. strain Zeid 14. The bacterium will be very useful for bioremediation of sites contaminated with molybdenum and amides.

Keywords: Molybdenum-reducing bacterium, molybdenum blue, *Bacillus* sp., acrylamide, acetonitrile

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INTRODUCTION

Manufacturing and prospecting exercises are the main contributors of molybdenum pollution of the natural environment. In Armenia, waste materials, seepages and effluents from the molybdenum-copper mine in Alaverdi have brought on the pollution of a territory approximately 300 square kilometres in size (Simeonov *et al.*, 2011). Metal and molybdenum mining activities in southern Colorado (USA) generated molybdenum pollution in surrounding areas with molybdenum level reaching 2,000 mg/L in the soils. The monitoring authority for e.g. The Water Quality Control Commission had stated that portions of about several miles of the Red River are so severely polluted that it is regarded as dead (LeGendre and Runnells, 1975). Molybdenum is also a by-product of a uranium mine in North Cave Hills South Dakota, (USA). At the most polluted site recorded in 2004, the molybdenum content of 6,550 mg kg^{-1} was reported (Stone and Stetler, 2008). Activities of oil shale exploration can also lead to molybdenum pollution. In Jordan, soils from such an exploration exhibited molybdenum concentration of 11.7 mg kg^{-1} (Al Kuisi *et al*., 2015). In Malaysia, molybdenum is produced on a small scale from the by-product of copper mining. The Mamut copper mine in Sabah, is an example. There are several reports on a number of cases of contamination caused by the accidental breakage of the metal-carrying pipe leading to episodic contamination of vast agricultural areas, which include the Ranau River (Kosaka and Wakita, 1978). In Batu Hijau, Sumbawa, Indonesia, copper-gold-molybdenum mining from the copper-gold-molybdenum porphyry deposit is recorded as being responsible for reduced fish population. As the mine dumps approximately several million tonnes of waste tailings into the ocean every year, a slow pollution of the sea is inevitable, and probably contributed for the reduced fish population observed (Apte and Kwong, 2003; Angel *et al.*, 2013).

Aside from chemical and physical removal of molybdenum, bacterial reductions into non-toxic forms have been suggested as alternatives, and include examples such as the reduction of soluble molybdate into either insoluble molybdenum disulphide (Tucker *et al.*, 1997) or to the colloidal molybdenum blue (Ghani *et al.*, 1993). Reduction to molybdenum disulphide requires fully anaerobic conditions, and two candidates that are often utilised for this purpose are *Desulfovibrio* and *Desulfotomaculum* (Biswas *et al*., 2009). Columns packed with polyacrylamide-immobilised sulphate-reducing bacteria have been attempted, and show reasonable success; however, the production of toxic hydrogen sulphide gas presents a challenge for such a process to be a commercial success (Tucker *et al.*, 1997). The reduction of molybdate to molybdenum blue is an alternative approach that has been recommended as a bioremediation tool (Ghani *et al*., 1993). Several advantages of this approach include observations such as the Mo-blue product being formed under low oxygen tension which is colloidal and does not pass through dialysis tubing easily, allowing some means of immobilisation and removal (Halmi *et al.*, 2014b). Molybdenum in the form of molybdate (sodium or ammonium salts) reduced to molybdenum blue is a phenomenon first mentioned in E. coli about more than one hundred years ago in 1896 (Capaldi and

Proskauer, 1896). This was followed by mention in the last century in 1939 (Jan 1939). It was reported again in 1985 in E. coli K12 (Campbell *et al.*, 1985), and in 1993 in *Enterobacter cloacae* strain 48 (Ghani *et al.*, 1993). The potential of this occurrence for use in the bioremediation of molybdenum was first realised by Ghani *et al.* (1993). Several bacterial genera that are potential candidates for bioremoval have been reported and include bacteria from the genera of *Bacillus* (Abo-Shakeer *et al.*, 2013; Othman *et al*., 2013), Klebsiella (Lim *et al*., 2012; Halmi *et al*., 2013; Masdor et al., 2015), *Acinetobacter* (Shukor et al., 2010b), *Pseudomonas* (Shukor *et al*., 2010a; Ahmad *et al.*, 2013), *Enterobacter* (Shukor *et al*., 2009c) and *Serratia* (Shukor *et al*., 2008a; Rahman *et al*., 2009; Yunus *et al.*, 2009; Shukor *et al.*, 2009d).

Amides and nitriles are toxic xenobiotics. An amide such as acrylamide is a monomer for polyacrylamide, a polymer. Amongst its many uses include as a sewage-flocculating agent, stabilising tunnels and dams, and as industrial adhesives (Rahim *et al.*, 2012). Acute cases of acrylamide toxicity has been reported causing death to fish and cows in Sweden (Svensson *et al*., 2003). The formulation of the pesticide glyphosate uses 20-30% polyacrylamide as a dispersing agent (Smith *et al.*, 1996), and this could be a substantial source of acrylamide pollution in soils and run-offs. Nitriles are cyanide-substituted carboxylic acids. Acetonitrile (CH_3CN) is a nitrile compound produced as a by-product of acrylonitrile. It is widely used as a precursor for various products. Acetonitrile is being produced to the tune of more than 40,000 tons annually (Hakansson *et al*., 2005). When ingested, acetonitrile is converted to toxic cyanide. Its pollution has been documented and research on its removal via bioremediation is being carried out globally. The acrylonitrile-acrylamide industries are known sources of acrylamide pollution with levels as high as 1 g/L have been reported (Rogacheva and Ignatov, 2001). Microbial degradation of amides and nitriles has been explored as a potential tool for their bioremediation. To date, numerous microorganisms have been isolated that are capable of degrading these compounds (Shukor *et al.*, 2009a; Shukor *et al.*, 2009b; Buranasilp and Charoenpanich, 2011; Rahim *et al*., 2012; Chandrashekar *et al.*, 2014).

Herein, a molybdenum-reducing bacterium with the novel capacity to use acrylamide as an electron donor for molybdenum reduction as well as able to use acrylamide, acetamide and acetonitrile as carbon sources for growth is reported. We also model the growth of the bacterium on these compounds using the modified Gompertz model and found the model can describe the growth curves adequately. The characteristics of this bacterium with its multiple detoxification capability are very useful for bioremediation.

MATERIALS AND METHODS

Isolation, Identification and Maintenance of Molybdenum-Reducing Bacterium

An industrially contaminated land in the city of Juba, South Sudan, Africa was the site of soil sampling carried out in 2012. Isolation of molybdenum-reducing

bacteria utilised a minimal salts media (MSM) with the phosphate concentration set at 5 mM and sodium molybdate at 10 mM. A soil suspension (1 g soil in 10 ml deionised water) was thoroughly mixed, and 0.1 mL of the soil suspension was then spread onto a petri dish containing agar media (w/v) as follows: $Na_2MoO_4 \cdot 2H_2O$ (0.242 % or 10 mM), yeast extract (0.5%), NaCl (0.5%), agar (1.5%), $MgSO_4 \cdot 7H_2O$ (0.05%), $(NH_4)_2 \cdot SO_4$ (0.3%), glucose (1%), and Na_2HPO_4 (0.071% or 5 mM). The pH of the media was adjusted to pH 6.5 (Masdor *et al.*, 2015). This media is known as a low phosphate molybdate media or LPM. After 48 h of incubation at room temperature, several white and ten blue colonies appeared on the plate. The ten isolates were repeatedly transferred onto other LPM agar plates to purify the bacteria. A quantification of the Mo-blue production was carried out in 100 mL liquid culture (LPM) to select the best isolate. Mo-blue production was quantified at 865 nm with an extinction coefficient of 16.3 mM^{-1} cm^{-1} to choose the best isolate. A scanning of the molybdenum blue absorption spectrum was carried out between 400 and 900 nm (UV-spectrophotometer, Shimadzu 1201), and LPM as the baseline correction. Identification of the Mo-reducing bacterium was carried out based on phenotypical and biochemical methods as outlined in Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994). The results were computed into the ABIS online system (Costin and Ionut, 2015).

Preparation of Bacterial Resting Cells

The characterisation of molybdenum reduction in a microtiter format including the concentrations of phosphate, molybdate, effects of carbon sources, pH, temperature and heavy metals were carried out at room temperature as done by Shukor and Shukor (2014). Briefly, bacterial cells were grown aerobically on an orbital shaker (120 rpm) in several 250 mL shake flasks in a volume of 1 L. The media utilised was the high phosphate media (HPM) with the only difference in the LPM being the setting of the phosphate concentration at 100 mM. This was carried out to prevent bacterial aggregations to molybdenum blue. Bacterial cells were first centrifuged at 10,000 g for 10 min at 4^{o} C. The bacterial pellets formed were rinsed twice with deionised water. A resuspension of the pellet in 20 mL of LPM was carried out next, but with glucose omitted. Suitable modifications to the LPM media were conducted to accommodate the various characterisation methods mentioned above. About 180 µL of the media was pipetted into the wells of a flat-bottom microplate. Sterile glucose (20 µL) was added to the final concentration of 1.0 % (w/v). The final volume was 200 µL. After the microplate was sealed, it was incubated at room temperature for two days. Mo-blue production was determined at 750 nm on a BioRad Microtiter Plate reader (Model No. 680, Richmond, CA). The extinction coefficient of 11.69 mM^{-1} cm^{-1} at 750 nm was utilised to quantify Mo-blue production (Shukor and Shukor, 2014). Heavy metals were sourced from Atomic Absorption Spectrometry standard solutions (Merck KGaA, Darmstadt, Germany).

Test of Amides and Nitriles as Sources of Electron Donor or Growth

The capacity of various amides and nitriles to aid molybdenum reduction was carried out by substituting glucose from the LPM media with nicotinamide, acetamide, iodoacetamide, acrylamide, propionamide, acetamide, acetonitrile, acrylonitrile 2-chloroacetamide, and benzonitrile to the final concentration of 1,000 mg/L (Arif *et al.*, 2013). Then 200 µL of the media was added into the microplate wells with 50 µL of resting cells suspension. The microplate was incubated at room temperature for three days and Mo-blue production was determined at 750 nm. The ability of the above compounds to support the growth of this bacterium was carried out by using the media below minus molybdate, and replacing glucose with the xenobiotics at 1,000 mg/L in a volume of 50 µL. The ingredients of the growth media (LPM) at pH 7.0 were as follows: $NaNO_3$ (0.2%), NaCl (0.5%), $MgSO_4 \bullet 7H_2O$ (0.05%), $(NH_4)_2 \bullet SO_4$ (0.3%), yeast extract (0.01%) and Na_2HPO_4 (0.705% or 50 mM). Then 200 µL of the media was added into the microplate wells and mixed with 50 µL of resting cells suspension. The increase in bacterial growth was measured at 600 nm after three days of incubation at room temperature.

Mathematical Modelling of Bacterial Growth on Zenobiotics

Bacterial growth on these xenobiotics was modeled using the modified Gompertz model (eqn. 1) as this model is frequently used to model microbial growth (Zwietering *et al.*, 1990).

$$y = A\exp\left\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t)+1\right]\right\} \qquad \text{(Equation 1)}$$

where A=bacterial growth at lower asymptote; μ_m = maximum specific bacterial growth rate, λ=lag time, *e* = exponent (2.718281828) and *t* = sampling time.

HPLC Analysis of Acrylamide Degradation

The confirmation of acrylamide degradation was carried out by HPLC analysis (Agilent,1100 series) with a manual Rheodyne™ sample injector of the degradation product, which is an acrylic acid production or producer?? (Shukor *et al.*,2009b) with slight modifications. Bacterial growth on acrylamide was carried out aerobically at room temperature for three days by shaking on an orbital shaker at 120 rpm. The media was the high phosphate media supplemented with 1,000 mg/L acrylamide in a 100 ml media. A suitable aliquot of the bacterial culture was centrifuged at 10,000 g for 10 min. A 20 µl sample filtered through a 0.45 µm polytetrafluoroethylene, (PTFE) filter, was injected into the HPLC system on a column (Supelco Discovery® HS C18, 4.6 x 150 mm, particle size 5 µm; Sigma-Aldrich Co., USA). The mobile phase was ultra pure water with a flow rate of 1 mL/min. Acrylic acid, analytical grade from Sigma (St; Louis U.S.A.) was utilised as a standard. Detection was performed at 196 nm using a reference wavelength of 360 nm.

RESULTS AND DISCUSSION

Numerous Mo-reducing bacteria have been reported including two SDS degraders (Halmi *et al*., 2013; Masdor *et al*., 2015) as well as an Antarctic isolate (Table 1). The microtiter plate format allows a high throughput characterisation format (Iyamu *et al.*, 2008; Shukor and Shukor 2014; Masdor *et al*., 2015). Characterisation the bacterium Enterobacter cloacae strain 48 (Ghani *et al.*, 1993) utilise resting cells or whole cells. Other bacterial characterisation works in metal reduction such as in chromate (Llovera *et al*., 1993), selenate (Losi and Frankenberger Jr., 1997) and vanadate (Carpentier *et al*., 2005) also capitalize on the use of resting cells. The use of resting cells bypasses the initial stage of the growth process that is normally affected by toxic xenobiotics.

TABLE 1
Characteristics of Mo-reducing bacteria isolated to date

Bacteria	Optimal Molybdate (mM)	Optimal Phosphate (mM)	Heavy metals inhibition	Optimal C source	Author
Klebsiella oxytoca strain Aft-7	5-20	5-7.5	Cu^{2+}, Ag^{+}, Hg^{2+}	glucose	(Masdor *et al.,* 2015)
Bacillus sp. strain A.rzi	50	4	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, $Hg^{2+,}$ Co^{2+},Zn^{2+}	glucose	(Othman *et al.,* 2013)
Bacillus pumilus strain lbna	40	2.5-5	As^{3+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	glucose	(Abo-Shakeer *et al.,* 2013)
Pseudomonas sp. strain DRY1	30-50	5	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, Hg^{2+}	glucose	(Ahmad *et al.,* 2013)
Klebsiella oxytoca strain hkeem	80	4.5	Cu^{2+}, Ag^{+}, Hg^{2+}	fructose	(Lim *et al.,* 2012)
Acinetobacter calcoaceticus strain Dr.Y12	20	5	Cd^{2+}, Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	glucose	(Shukor *et al.,* 2010b)
S. marcescens strain Dr.Y9	20	5	Cr^{6+}, Cu^{2+}, Ag^{+}, Hg^{2+}	sucrose	(Yunus *et al.,* 2009)
Serratia sp. strain Dr.Y8	50	5	Cr, Cu, Ag, Hg	sucrose	(Shukor *et al.,* 2009d)
Pseudomonas sp. strain DRY2	15-20	5	Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	glucose	(Shukor *et al.,* 2010a)
Serratia sp. strain Dr.Y5	30	5	n.a.	glucose	(Rahman *et al.,* 2009)
Enterobacter sp. strain Dr.Y13	25-50	5	Cr^{6+}, Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}	glucose	(Shukor *et al.,* 2009c)
Serratia marcescens strain DRY6	15-25	5	Cr^{6+}, Cu^{2+}, Hg^{2+}	sucrose	(Shukor *et al.,* 2008a)
Enterobacter cloacae strain 48	20	2.9	Cr^{6+}, Cu^{2+}	sucrose	(Ghani *et al.,* 1993)
Escherichia coli K12	80	5	Cr^{6+}	glucose	(Campbell *et al.,* 1985)

Ten Mo-reducing bacterial isolates were determined for their molybdenum blue production in LPM media. The best isolate was isolate g (Table 2), and was chosen for further studies. Bacillus sp. strain Zeid 14 was incubated at different initial pH ranging from 5.5 to 8.0. The optimum pH for Mo-blue production occurred between 6.0 and 6.8 (Figure 1). Optimum temperature for molybdenum reduction was between 25° C and 34° C (Figure 2). Mo-blue production was inhibited by the heavy metals of mercury, silver, copper, chromium and cadmium at 2 mg/L by 82.9, 68.5, 62.9, 27.3 and 10.2 %, respectively (Figure 3). The target for heavy metals mercury and copper inhibition of bacterial chromate reduction, another similar anionic metal, is the sulfhydryl group as this is a common target for toxic metal ions (Rege *et al.*, 1997; Elangovan *et al*., 2010), and could probably be the same as in molybdenum reduction. Sites containing these toxic cationic metal ions can be modified by adding calcium carbonate, phosphate, manganese oxide, thiosulphate, sulphur, and magnesium hydroxide to reduce the bioavailability and mobility of these toxic cationic metal ions (Hettiarachchi *et al.*, 2000; Deeb and Altalhi 2009). This action could increase the efficacy of molybdenum bioremediation despite the presence of the above mentioned toxic metal ions.

TABLE 2
Mo-blue production by bacterial isolates, measured at 750 nm (±standard deviation of triplicate).

Isolate	A 750 nm		
a	0.35	±	0.01
b	0.35	±	0.02
c	0.71	±	0.03
d	1.01	±	0.07
e	1.02	±	0.06
f	0.71	±	0.01
g	1.81	±	0.08
h	0.71	±	0.01
i	1.06	±	0.02
j	0.35	±	0.02

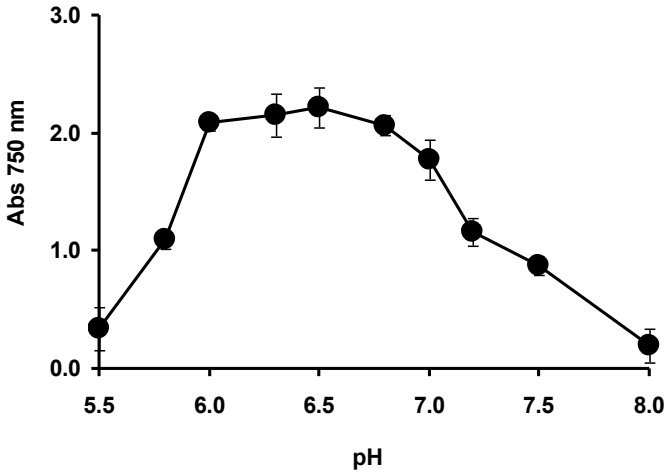

Figure 1: The effect of pH on molybdenum (molybdate) reduction to molybdenum blue. The error bars signify the standard deviation of the average of triplicate experiments.

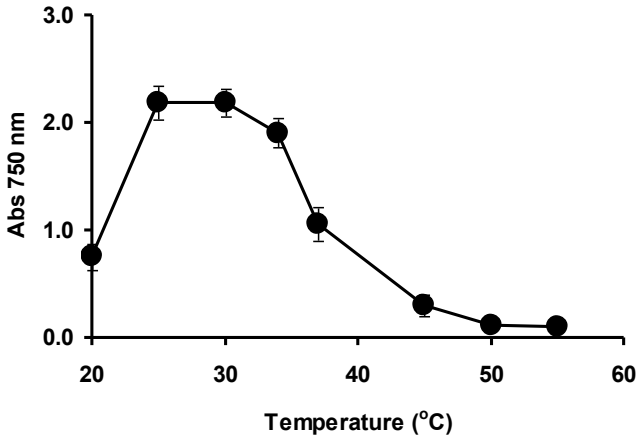

Figure 2: The effect of temperature on molybdenum (molybdate) reduction to molybdenum blue. The error bars signify the standard deviation of the average of triplicate experiments.

Molybdenum-reducing Bacterium Identification

Isolate g was a gram-positive, rod-shaped bacterium. The results of morphological and various biochemical tests are presented in Table 3. The ABIS online software suggested *Bacillus subtilis* as the bacterial identity with a homology score of 91% and accuracy at 92%. Despite this, a more accurate identification method employing 16s rRNA phylogenetic analysis is being conducted to identify this species further. However, at this moment, the bacterium is identified as Bacillus sp. strain Zeid 14.

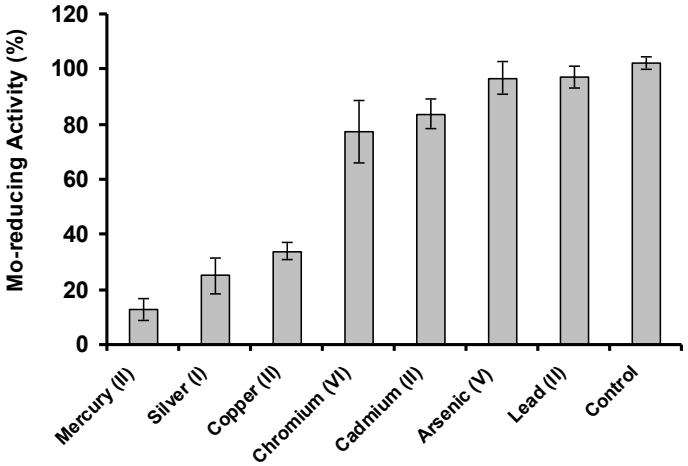

Figure 3: The effect of heavy metals on molybdenum (molybdate) reduction to molybdenum blue. The error bars signify the standard deviation of the average of triplicate experiments.

TABLE 3
Biochemical tests for *Bacillus* sp. strain Zeid 14

		Acid production from	
Gram positive staining	+		
Motility	+	N-Acetyl-D-Glucosamine	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	–	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 (ADH)	–	Ribose	+
Lysine decarboxylase (LDC)	–	Salicin	+
Ornithine decarboxylase (ODC)	–	Sorbitol	+
Citrate utilization	+	Sucrose	+
Egg-yolk reaction	–	Starch	+
Nitrates reduction	+	Trehalose	+
Voges-Proskauer test (VP)	+	D-Xylose	+

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

Molybdenum Blue Absorption Spectrum

The Mo-blue produced exhibited a spectrum with a maximum peak at 865 nm, and a shoulder at 700 nm. This unique spectrum was observed at various incubation periods (Figure 4). Prior to scanning analysis, samples were centrifuged at 10,000 g for 10 min. As the incubation period increased, the particular profile was preserved. An earlier proposal for the mechanism of molybdenum reduction in microorganism suggests an initial requirement of an enzymatic reduction from Mo^{6+} to Mo^{5+} state. This is followed by a second reaction involving phosphate ions addition that produces Mo-blue (Ghani *et al*., 1993). Despite this, the reaction does not conform to molybdate chemistry as the acidic conditions of bacterial fermentation of glucose or sucrose quickly convert molybdate anions into polymolybdates. In the presence of phosphate, a phosphomolybdate is formed. This will then be followed by bacterial reduction of phosphomolybdate into Mo-blue, and this is another mechanism that we previously proposed (Shukor *et al.*, 2007). The involvement of phosphomolybdate during bacterial reduction of molybdenum can be inferred from the Mo-blue spectrum obtained. The Mo-blue spectra seen in this work, and from nearly all of the isolated Mo-reducing bacteria (Shukor *et al.,* 2007; Masdor *et al*., 2015) showed similar spectra to the phosphate determination method (PDM), which has been confirmed to be a reduced phosphomolybdate (Clesceri *et al*., 1989; Chae *et al*., 1993; Shukor *et al.*, 2007), supporting the second mechanism. The presence of an unstable intermediate species, Cr^{5+} during bacterial reduction of Cr^{6+} to Cr^{3+} has been confirmed using electron paramagnetic and UV-spectroscopic studies (Cervantes *et al*., 2001), indicating that the existence of an intermediate species is not unique to molybdenum. This was observed in the *Shewanella putrefaciens* (Myers *et al*., 2000) and *Pseudomonas ambigua* (Suzuki *et al*., 1992). Further evidence in support of the second mechanism is seen in the reduction of phosphomolybdate, but not molybdate, by the enzymes xanthine and aldehyde oxidases to Mo-blue (Glenn and Crane, 1956). The process of the formation of phosphomolybdate from molybdate is chemically complicated. In acidic pHs, and at molybdate concentrations over 1 mM, molybdate ions are instantaneously converted to polyions such as $H_2Mo_7O_{24}^{4-}$, $HMo_7O_{24}^{5-}$, $Mo_7O_{24}^{6-}$, and $Mo_{12}O_{37}^{2-}$. As the solution is acidified to pH of less than 2.0, species such as $Mo_8O_{26}^{4-}$ and $Mo_{36}O_{112}(H_2O)_{16}^{8-}$ start to form. All of these forms of molybdenum are called polyoxomolybdates, which can further incorporate heteroatoms such as silicate or phosphate forming the heteropolyoxomolybdates such as silicomolybdate and phosphomolybdate (Krishnan *et al*., 2008). The species of phosphomolybdate formed in the phosphate determination method is $PMo_{12}O_{40}^{3-}$, with α-Keggin structure. This can be reduced further by a reducing agent, for example, ascorbic acid to form the β-keggin ion, $PMo_{12}O_{40}^{7-}$, which is known as molybdenum blue (Barrows *et al.*, 1985). Phosphomolybdate can exist in the form of several lacunary species. In addition, these lacunary species can interchange, subject to environmental change in pH. This makes accurate identification difficult, and requires nuclear magnetic resonance and electron spin resonance methods. Fortunately, spectroscopic

analysis has been proposed as a suitable and simple method for distinguishing between the heteropolymolybdates such as silicomolybdate, phosphomolybdate, and sulfomolybdate (Sims, 1961; Chae *et al*., 1993).

Carbon Sources as Electron Donor for Molybdenum Reduction

Mo-blue production was optimally supported by glucose in descending efficiency by sucrose, fructose, lactose, maltose, glycogen, d-mannose, trehalose, meso-inositol and glycerol (Figure 5). The concentration of glucose optimally supporting molybdenum reduction was 1% (w/v) with higher concentrations inhibiting reduction (Figure 6). The inhibition seen is probably due to osmotic stress as discussed previously (Shukor *et al*., 2009c). Many metal bacterial reductions also utilise glucose effectively. This is seen in selenate (Losi and Frankenberger Jr., 1997), vanadate (Antipov *et al.*, 2000), chromate (Llovera *et al.*, 1993), and arsenate (Chang *et al*., 2012) reductions. Nearly all of the Mo-reducing bacteria either prefer glucose or sucrose as the best electron donor (Table 1). Bacterial reduction, including molybdate reduction, require NADH and/or NADPH as electron donating substrates. These compounds are efficiently generated with assimilable sugars such as glucose or sucrose using metabolic pathways that include the glycolytic pathway in anaerobic conditions, and the citric acid cycle and the electron transport chain under aerobic conditions or in the presence of suitable terminal electron acceptors (Llovera *et al.*, 1993; Losi and Frankenberger Jr., 1997; Antipov *et al.*, 2000; Shukor *et al.*, 2008b; Chang *et al.*, 2012; Shukor *et al.*, 2014). Cheaper and more available carbon sources such as molasses that are plentiful in Sudan (as a sugarcane waste product) may be used in the future (Medjeber *et al.*, 2015).

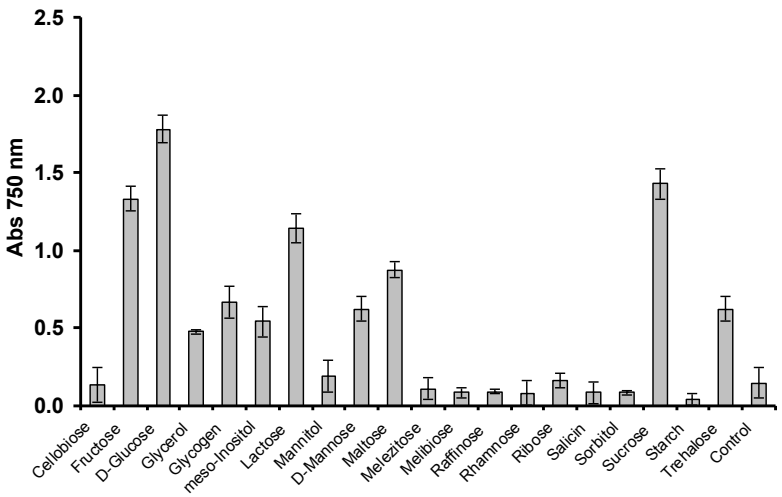

Figure 5: The effect of various carbon sources as electron donor for molybdenum (molybdate) reduction to molybdenum blue. The error bars signify the standard deviation of the average of triplicate experiments.

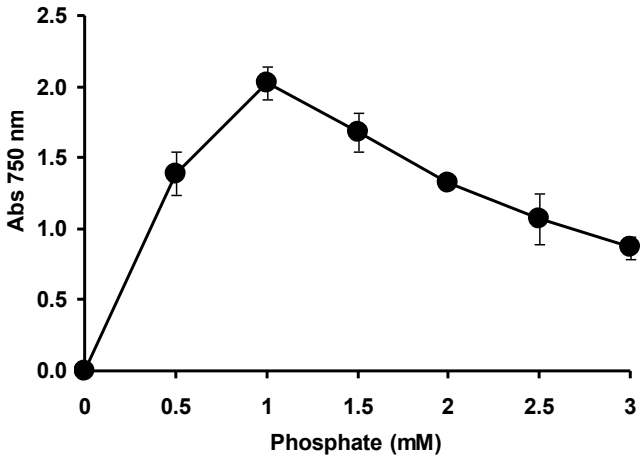

Figure 6: The effect of glucose concentration as electron donor for molybdenum (molybdate) reduction to molybdenum blue. The error bars signify the standard deviation of the average of triplicate experiments.

Molybdenum Reduction at Various Phosphate and Molybdate Concentrations

Mo-blue production was supported optimally by phosphate concentration at 5 mM. Higher concentrations were detrimental to Mo-blue production (Figure 7) while the optimal molybdate concentration for reduction was between 10 and 20 mM (Figure 8). It is observed generally, that phosphate concentrations beyond 5 mM inhibited molybdate reduction in nearly all Mo-reducing bacteria isolated to date (Table 1). The compound or complex phosphomolybdate is highly unstable at neutral pH, and is rapidly oxidised (Glenn and Crane 1956). At concentrations of phosphate of 20 mM and beyond, the pH is maintained strongly at neutrality causing the rapid destabilisation of phosphomolybdate. In addition, phosphate by itself can destabilise the phosphomolybdate complex. This is observed in a study, where an acidified phosphate solution at 100 mM leads to the destabilisation of Mo-blue from an ascorbate-reduced phosphomolybdate (Shukor *et al.*, 2002). The concentrations of molybdate supporting optimal Mo-blue production in nearly all of the bacteria isolated to date range between 5 and 80 mM (Table 1). In contrast to cationic heavy metals, bacteria can tolerate and reduce high concentrations of anionic heavy metals. For instance, the most tolerant microorganism can tolerate and reduce chromate at 30 mM in *Pseudomonas putida* (Keyhan *et al.*, 2003), selenate at 20 mM in *Bacillus* sp. (Fujita *et al.*, 1997), arsenate at 60 mM in *Citrobacter* sp. NC-1 (Chang *et al*., 2012) and vanadate at 50 mM in *Pseudomonas isachenkovii* (Antipov *et al*., 2000). Thus, these bacteria can be efficient candidates for remediation of molybdenum-polluted areas. In Colorado, contaminated sites from a discontinued uranium mine displayed molybdenum concentrations as much as 6,500 mg k^{g-1} and 900 mg L^{-1} in soils and water, respectively (Stone and Stetler 2008). Based on the inhibition of phosphate, remediation of contaminated sites that have phosphate above 20 mM will be severely affected. Most type of

soils and water bodies have phosphate concentrations far lower than this value, and bioremediation of molybdenum utilising Mo-reducing bacteria would not be affected (Jenkins 1973).

Mo-blue Production and Bacterial Growth on Amides and Nitriles

The ability of these amides and nitriles to support molybdenum reduction was explored. Of all the xenobiotics tested, acrylamide, propionamide and acetamide were shown to support molybdenum reduction while other xenobiotics tested could not (Figure 9). The amides acrylamide, acetamide and acetonitrile could support the growth of this bacterium independently of molybdenum reduction (Figure 9). The growth of this bacterium on the compounds was modelled according to the modified Gompertz model (Figure 10). The absorbance values at 600 nm were first converted to natural logarithm. The growth parameters obtained were lag periods of 0.468, 0.979 and 1.53 d and at maximum specific growth rates of 1.165, 0.932, 0.842 d^{-1} for acrylamide, acetamide and acetonitrile respectively. The correlation coefficients obtained for the model at 0.99, 0.98 and 0.98 for acrylamide, propionamide and acetamide, respectively, indicated good agreement between predicted and observed values. HPLC analysis indicated a lowering of acrylamide concentration and the presence of the metabolite acrylic acid at the end of incubation period (Figure 11). Acrylic acid is also detected as a metabolite in previously published results on acrylamide-degrading microorganism (Shukor *et al.*, 2009a; Shukor *et al.*, 2009b; Rahim *et al*., 2012).

This is the first report on carbon sources other than carbohydrates that could support Mo- reduction in bacterium. In chromate reduction, xenobiotics such as

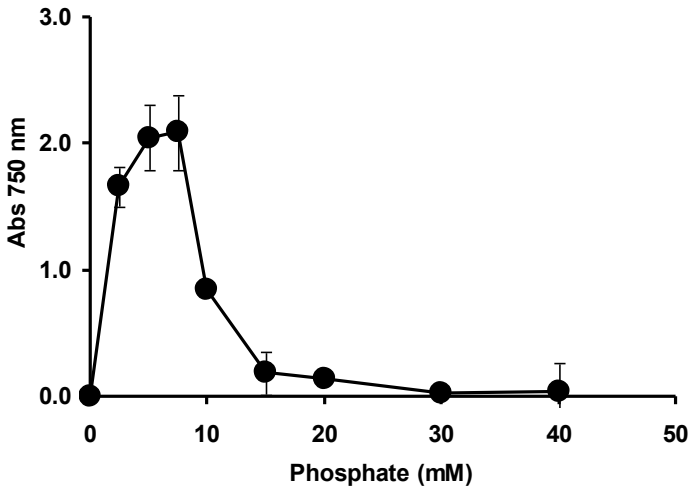

Figure 7. Molybdenum blue production at various concentrations of phosphate. The concentration of molybdate was at 10 mM. The error bars signify the standard deviation of the average of triplicate experiments.

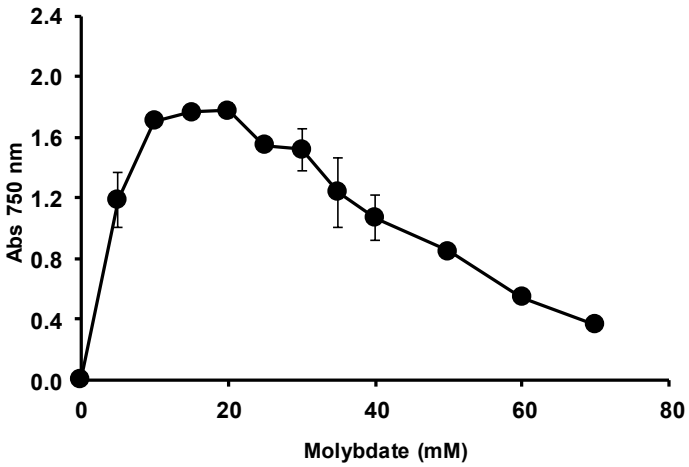

Figure 8. Molybdenum blue production at various concentrations of sodium molybdate. The concentration of phosphate was at 5 mM. The error bars signify the standard deviation of the average of triplicate experiments.

phenol is used as electron donors (Anu *et al.*, 2010). Amides such as acrylamide, acetamide and propionamide are produced in the order of millions of tonnes per year. Several microbes have been isolated that could use these amides and nitriles as carbon or nitrogen sources including *Stenotrophomonas acidaminiphila* MSU1 (Lakshmikandan *et al.,* 2014), *Variovorax boronicumulans* CGMCC 4969 (Liu *et al.*, 2013), *Pseudomonas azotoformans* (Komeda *et al*., 2004), *Pseudomonas aeruginosa* (Chandrashekar *et al.*, 2014), *Pseudomonas putida* (Nawaz *et al.*, 1989), *Pseudomonas acidovorans* (Alt *et al*., 1975), *Pseudomonas* sp. (Shukor *et al*., 2009b) and *Pseudomonas chlororaphis* (Ciskanik *et al*., 1995). Other acrylamide-degrading bacteria that have been reported include *Bacillus cereus* (Halmi *et al.*, 2014a), *Pseudonocardia thermophilia, Thermococcus hydrothermalis* (Postec *et al*., 2005), *Rhodococcus* sp. (Nawaz *et al*., 1998), *Burkholderia* sp. (Syed *et al*., 2012), *Enterobacter aerogenes* (Buranasilp and Charoenpanich, 2011), *Kluyvera georgiana* (Thanyacharoen *et al*., 2012) and the yeast *Rhodotorula* sp. (Rahim *et al*., 2012).

CONCLUSION

Molybdenum reduction to the colloidal molybdenum blue is a potential candidate for molybdenum bioremediation. We have isolated a Mo-reducing bacterium which can utilise acrylamide, an amide, as a source of electron donor. In addition, the amides acrylamide, acetamide and acetonitrile could support the growth of this bacterium. A modified Gompertz model was successfully used to model the growth of the bacterium on these compounds, and important growth parameters were obtained. Reduction of molybdenum required a narrow phosphate concentration and glucose as an electron donor. Mo-blue production requires a strict phosphate concentration of between 5.0 and 7.5 mM. The

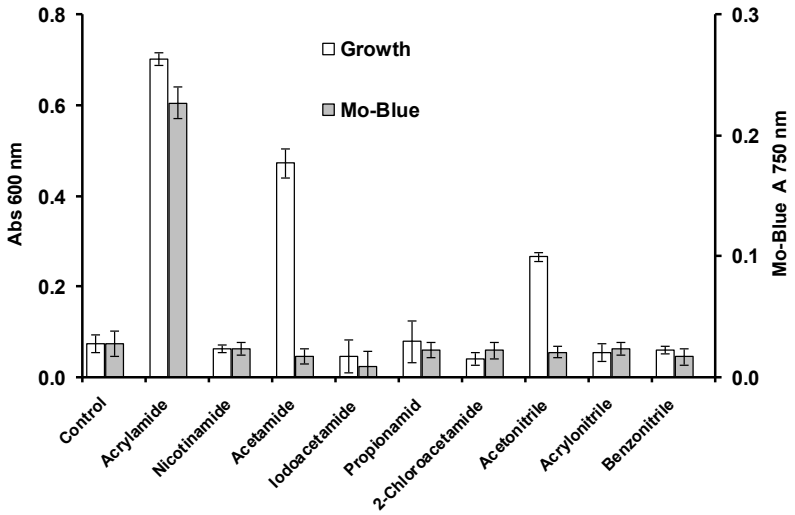

Figure 9. Mo-blue production and growth of Bacillus sp. strain Zeid 14 on various xenobiotics. The error bars signify the standard deviation of the average of triplicate experiments.

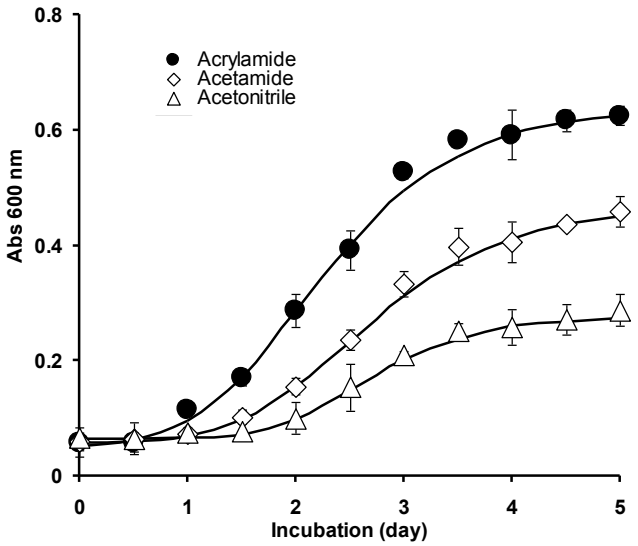

Figure 10. Growth of Bacillus sp. strain Zeid 14 on acrylamide (●) acetamide (◊) and acetonitrile (Δ) at 1,000 mg/L modelled using the modified Gompertz model (solid lines). Growth was carried out aerobically in a 50 mL media at room temperature on an orbital shaker (120 rpm). The error bars signify the standard deviation of the average of triplicate experiments.

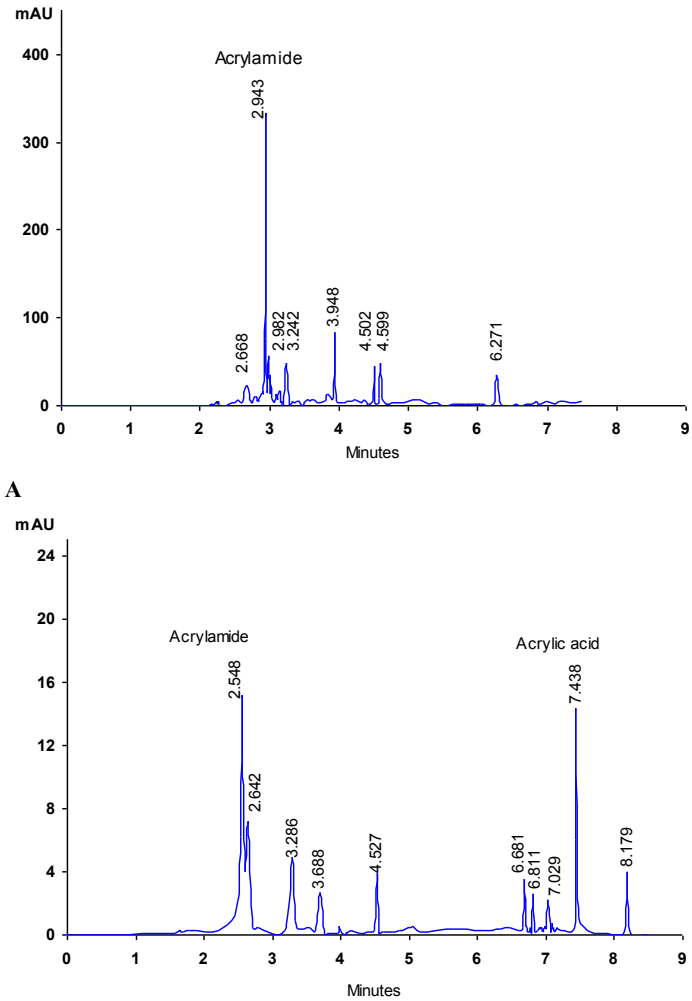

Figure 11: HPLC chromatogram of acrylamide at the start of incubation (A) and acrylic acid detection during acrylamide degradation (B).

scanning absorption spectrum results indicate that the identity of the Mo-blue is likely a reduced phosphomolybdate. Toxic cationic heavy metals inhibited Mo-blue production. The ability of this bacterium to detoxify many toxicants is very useful for bioremediation. Current works include a further characterisation of the xenobiotics-degrading properties, purification of the Mo-reducing enzyme and a further identification of the bacterium via molecular techniques.

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