

ISOLATION AND CHARACTERIZATION OF A MOLYBDENUM-REDUCING AND PHENOLIC- AND CATECHOL-DEGRADING *Enterobacter* sp. STRAIN SAW-2

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ABSTRACT

Molybdenum is an emerging pollutant worldwide. The objective of this study is to isolate molybdenum-reducing bacterium with the ability to grow on phenolic compounds (phenol and catechol). The screening process was carried out on a microplate. The bacterium reduced molybdenum in the form of sodium molybdate to molybdenum blue (Mo-blue). The bacterium required a narrow pH range for optimal reduction of molybdenum, i.e. between pH 6.3 and 6.8, with temperature between 34 and 37 °C. Molybdate reduction to Mo-blue was best supported by glucose as the carbon source. However, both phenol and catechol could not support molybdate reduction. Other requirements for molybdate reduction included sodium molybdate concentrations between 15 and 30 mM, and phosphate concentration of 5.0 mM. The bacterium exhibited a Mo-blue absorption spectrum with a shoulder at 700 nm and a maximum peak near the infrared region at 865 nm. The Mo-reducing bacterium was partially identified as *Enterobacter* sp. strain Saw-2. The capability of this bacterium to grow on toxic phenolic compounds and to detoxify molybdenum made it a significant agent for bioremediation.

Keywords: Catechol, *Enterobacter* sp., Molybdenum, molybdenum blue, phenol

INTRODUCTION

Heavy metals are toxic to organisms. Some microorganisms are able to detoxify heavy metals by employing mechanisms such as biosorption, sequestration or chelation, active pumping and reduction (Batta *et al.* 2013; Oves *et al.* 2013; Banerjee *et al.* 2016). Microorganisms having capability to reduce heavy metals such as chromium, molybdenum mercury into less toxic forms were documented. Molybdenum (Mo) is essential to organisms as it is a cofactor to many important enzymes such as nitrogenase, sulfite oxidase, aldehyde oxidase and xanthine oxidoreductase (Daniels *et al.* 2008; Leimkühler *et*

al. 2011). Molybdenum is not generally toxic to human. However, molybdenum is very toxic to ruminants, such as sheep and cattle at concentrations as low as several parts per million (ppm). An elevated level of molybdenum in ruminants causes a disease known as hypercuprosis (Ward 1978). Recent data showed that molybdenum is toxic to spermatogenesis and embryogenesis in animals, including catfish and mice (Meeker *et al.* 2008; Bi *et al.* 2013; Zhai *et al.* 2013; Zhang *et al.* 2013). Toxic effect of molybdenum to these animals warrants removal of molybdenum from the environment. Heavy usage of molybdenum in various steel products and lubricants resulted in high concentration of molybdenum reaching hundreds of ppm in the water bodies of the Black Sea and Tokyo Bay

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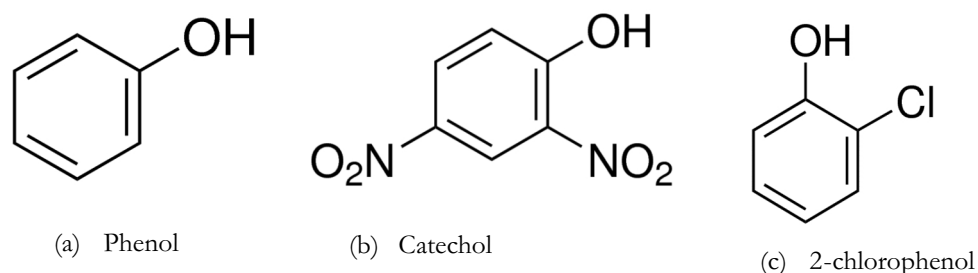


Figure 1 Chemical structure of some toxic phenolic compounds

(Davis 1991; Neunhäuserer *et al.* 2001). Terrestrially, in the mine tailings of a molybdenum mine in New Mexico, as high as 2,000 ppm of molybdenum concentration were documented (Runnells *et al.* 1976). Together with heavy metals, oil, grease and phenolics are hydrocarbon which are reported to be the number one scheduled waste (Yadzir *et al.* 2016). Phenol and phenolic compounds (Fig. 1) are not only toxic to human, but also to many other organisms (Shukor *et al.* 2008a; Shukor *et al.* 2008b; Rahman *et al.* 2009; Yunus *et al.* 2009).

The presence of multiple pollutants requires microorganisms with multiple detoxification ability. Thus, the objective of this study is to isolate such a microorganism. In this study, we reported on the isolation of a molybdenum-reducing bacterium with the ability to grow on phenolic compounds (phenol and catechol).

MATERIALS AND METHODS

Chemicals

Chemicals such as $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $(\text{NH}_4)_2\text{SO}_4$, NaCl and Na_2HPO_4 were purchased from Sigma Aldrich (St. Louis, MO, USA) and were of analytical grade while glucose and yeast extract were purchased from Fisher (Malaysia).

Isolation of Molybdenum-Reducing Bacterium

Molybdenum-reducing bacterium was isolated using two kinds of media, i.e. low phosphate molybdate (LPM) and high phosphate molybdate (HPM) media. LPM medium consists of the following composition: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05% w/v), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.206% or 10 mM), yeast extract (0.05% w/v), $(\text{NH}_4)_2\text{SO}_4$ (0.3% w/v),

glucose (1% w/v), NaCl (0.5% w/v) and Na_2HPO_4 (0.071% w/v or 5 mM). The LPM medium was adjusted to pH 7.0. Agar having concentration of 1.5% (w/v) was added to the LPM solid media. Bacterial cell harvesting from the LPM medium could not be carried out, as blue aggregates formed in the LPM liquid media. Hence, the phosphate concentration was increased to 100 mM which made the medium became HPM (Ghani *et al.* 1993). Soil materials for the isolation of Molybdenum-reducing bacteria were taken from topsoil depth of 5 cm in a polluted area in Kuching, Sarawak (1.6077° N, 110.3785° E) in April, 2011. A cell suspension containing 1 g of soil and 10 mL of sterile distilled water was prepared. Soil suspension of 0.1 mL aliquot was immediately spread on an agar plate containing LPM media. The plates were incubated for 48 hours at room temperature (27 °C). Several white and blue colonies appeared afterwards. Colony with the strongest blue intensity was restreaked on LPM agar until a pure culture was obtained. HPM media was utilized to prepare for the resting cells of the molybdenum-reducing bacterium. Growth of the bacterium was carried out in a 1 L culture volume, shaken using orbital shaker, set at 120 rpm, and incubated for 48 hours at room temperature (27 °C). Cells were harvested via centrifugation (10,000 xg, for 10 minutes). Distilled water was utilized to wash the bacterial pellets after centrifugation twice. The pellets were then resuspended in 10 mL of LPM media (5 mM) with the glucose omitted.

Preparation of Resting Cells

Preparation and use of resting cells in a microtiter format were carried out based on Shukor and Shukor (2014). Cell suspension prepared previously (180 mL) was transferred to each well of a sterile microplate. Sterile glucose (20 mL) from a 10% (w/v) stock solution was

mixed with the cell suspension. The microplate was covered with a sterile sealing tape (Corning® microplate). The microplate was incubated at room temperature (27 °C) for 48 hours. Mo-blue production was determined at 750 nm in a microtiter plate reader Model No. 680, (BioRad, Richmond, CA). The specific extinction coefficient of $11.69 \text{ mM}^{-1}\text{cm}^{-1}$ was utilized to determine Mo-blue concentration (Shukor *et al.* 2003). Heavy metals such as lead (II), arsenic (V), mercury (II), silver (I), chromium (VI) copper (II) and cadmium (II) on Mo-blue production were obtained from Atomic Absorption Spectrometry (AAS) calibration solutions (Merck Chemical Co., Germany). The capability of phenolics to act as electron donors was tested using the microplate format. The phenolics tested were phenol, 2,4-dinitrophenol, pentachlorophenol, 2-chlorophenol, 4-chlorophenol, catechol, salicylic acid, 4-nonylphenol, p-hydroxybenzoic acid, benzoate and 2-naphthol. The phenolics replaced glucose and were tested at 200 mg/L, but in a volume of 50 mL (Arif *et al.* 2013). If the phenolics could be used as electron donors, Mo-blue production will increase. On the other hand, ability of the above phenolics to support the growth of this bacterium independently from molybdenum-reduction was carried out in HPM media minus molybdenum. The increase of bacterial growth after 48 hours of incubation at room temperature, which was an indication of phenolics assimilation, was measured at 600 nm.

Identification of the Molybdenum-Reducing Bacterium

Standard biochemical tests according to the Bergey's Manual (Holt *et al.* 1994) was used to identify the Molybdenum-reducing bacterium. In addition, a software-based method (ABIS online system) was utilized to interpret the results (Costin & Ionut 2015). Briefly, the standard methods included gram staining, detection of motility via the hanging drop method, and various biochemical tests.

Statistical Analysis

Data analyses were carried out using Graphpad Prism v 6.0 (www.graphpad.com). The Student's t-test or ANOVA with Tukey's test as the post hoc analysis was carried out to compare means of groups.

RESULTS AND DISCUSSION

Bacterial molybdenum reduction to Mo-blue is a phenomenon that has been described for more than one hundred years and is a prospective bioremediation tool (Shukor *et al.* 2014). This phenomenon was initially observed in 1896 in *E. coli* bacterium (Levine 1925). Comprehensive research about this phenomenon was only started in 1985 in the *E. coli* K12 bacterium (Campbell *et al.* 1985). Later on, sodium molybdate reduction into Mo-blue by the *Thiobacillus ferrooxidans* bacterium, a chemolithotroph, was reported by Sugio *et al.* (1988) without mentioning the works of Campbell *et al.* (1985). This suggested the scarcity of publications on this phenomenon. *Enterobacter cloacae* strain 48 (EC 48) is the first bacterium isolated from the Malaysian soils with the capacity to reduce molybdate (Ghani *et al.* 1993). The first Molybdenum-reducing bacterium reported with the ability to detoxify other xenobiotics is the SDS-degrading *Klebsiella oxtoca* (Halimi *et al.* 2013). Isolation of more xenobiotics-detoxifying Molybdenum-reducing bacterium can expand the capability of existing isolates for bioremediation of sites containing molybdenum, together with other xenobiotics.

Partial Identification of the Molybdenum-Reducing Bacterium

The bacterium was Gram-negative, rod-shaped and motile. The biochemical test results using the ABIS online software showed that bacterium having the highest homology (90%) and the highest accuracy (97%) was *Enterobacter cloacae* (Table 1).

The bacterium is tentatively named *Enterobacter* sp. strain Saw-2. Identification of the bacterium up to the species level can only be carried out through the addition of several more polyphasic methods, such as 16S rRNA gene sequencing, DNA-DNA hybridization determination of genomic DNA G+C content and fatty acid profile (Ahmad *et al.* 2013). Two molybdenum-reducing bacteria from this genus, i.e. *Enterobacter cloacae* strain 48 (Ghani *et al.* 1993) and *Enterobacter* sp. strain Dr.Y13 (Shukor *et al.* 2009) were previously reported. A bacterium from this genus has been reported for having ability to tolerate high concentrations of heavy metals such as nickel, cadmium and lead at levels of up to 700,

Table 1 Biochemical tests for *Enterobacter* sp. strain Saw-2

Motility	+	Acid production from:	
Pigment	-	Alpha-Methyl-D-Glucoside	+
Catalase production (24 h)	+	D-Adonitol	+
Oxidase (24 h)	-	L-Arabinose	+
ONPG (beta-galactosidase)	+	Cellobiose	+
Arginine dihydrolase (ADH)	+	Dulcitol	d
Lysine decarboxylase (LDC)	-	Glycerol	+
Ornithine decarboxylase (ODC)	+	D-Glucose	+
Nitrates reduction	+	myo-Inositol	+
Methyl red	d	Lactose	+
Voges-Proskauer (VP)	+	Maltose	+
Indole production	-	D-Mannitol	+
Hydrogen sulfide (H ₂ S)	-	D-Mannose	+
Acetate utilization	+	Melibiose	+
Malonate utilization	+	Mucate	+
Citrate utilization (Simmons)	+	Raffinose	+
Tartrate (Jordans)	+	L-Rhamnose	+
Esculin hydrolysis	+	Salicin	+
Gelatin hydrolysis	d	D-Sorbitol	+
Urea hydrolysis	+	Sucrose (Saccharose)	+
Deoxyribonuclease	-	Trehalose	+
Lipase (com oil)	-	D-Xylose	+
Phenylalanine deaminase	-		

Notes: + = positive result; - = negative result; d = indeterminate result

900 and 1,100 ppm, respectively (Banerjee *et al.* 2015). This phenomenon suggested that being heavy metal tolerant and conducting heavy metal reduction are part of the strategies employed by bacteria from this genus to combat heavy metal

toxicity. Nickel was detected to be more toxic (700 ppm), followed by cadmium (900 ppm) and lead (1,100 ppm). Optimal pH that supported molybdenum reduction was between 6.5 and 6.8 (Fig. 2), in temperature range of 34 - 37 °C (Fig. 3).

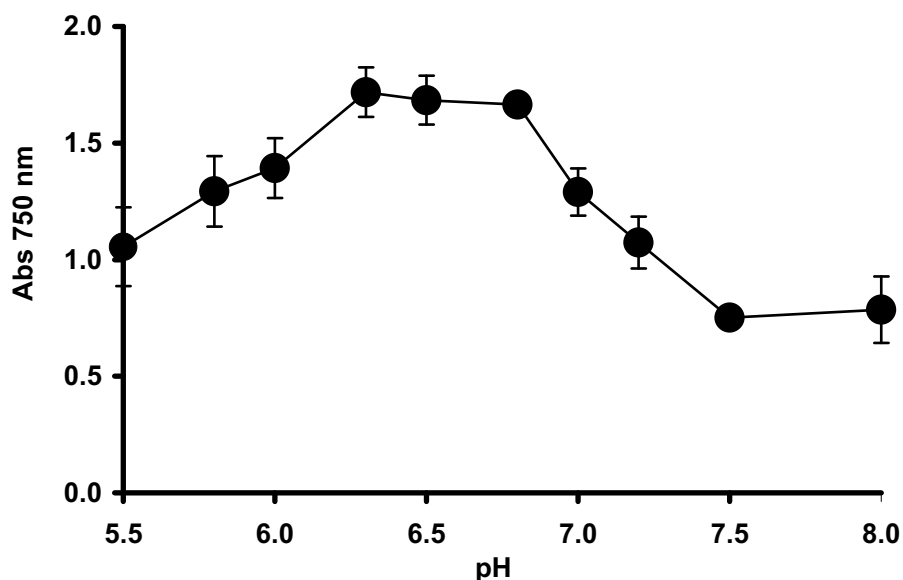


Figure 2 Effect of initial pH on molybdenum reduction by *Enterobacter* sp. strain Saw-2

Note: Error bars represent mean \pm standard deviation (n = 3)

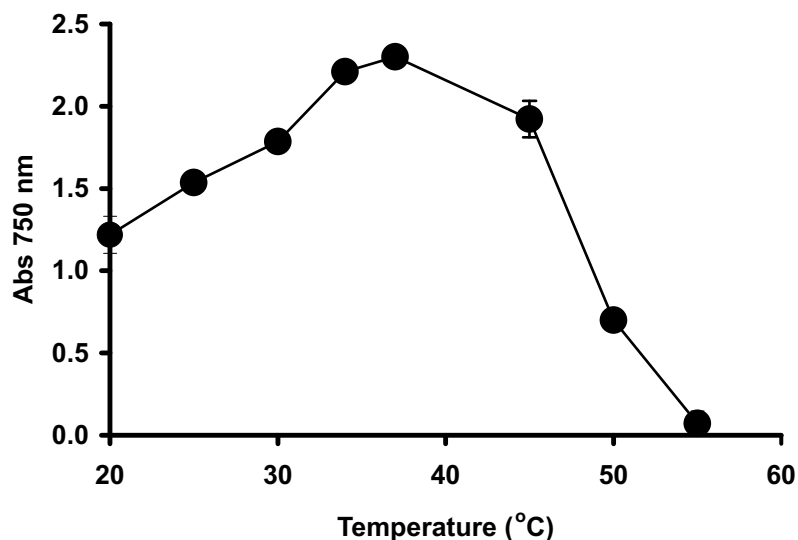


Figure 3 Effect of temperature on molybdenum reduction by *Enterobacter* sp. strain Saw-2
 Note: Error bars represent mean \pm standard deviation (n = 3)

High-throughput method using a microplate format was employed in this study. This method can accelerate characterization works, while acquiring more information as opposed to the regular shake-flask method (Iyamu *et al.* 2008; Shukor & Shukor 2014). The use of resting cells for studying Mo-blue production was initiated in EC 48 (Ghani *et al.* 1993). Resting cells were also used in studying heavy metals reduction such as in chromate (Llovera *et al.* 1993), selenate (Losi & Jr

1997) and xenobiotics biodegradation such as diesel (Auffret *et al.* 2015) and phenol (Sedighi & Vahabzadeh 2014).

Molybdenum Absorbance Spectrum

The scanning absorption spectrum of the resultant Mo-blue from *Enterobacter* sp. strain Saw-2 showed a maximum peak at 865 nm, and a characteristic shoulder at about 700 nm (Fig. 4).

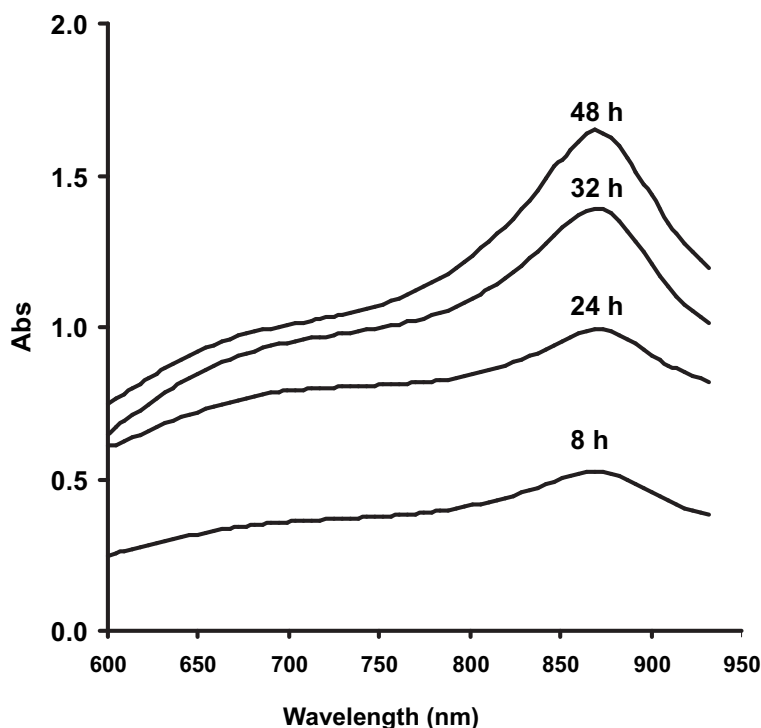


Figure 4 Scanning absorption spectrum of Mo-blue from *Enterobacter* sp. strain Saw-2 at different time intervals

It was observed that the resulting spectrum resembled the spectrum of the Mo-blue produced by the phosphate determination method (Clesceri *et al.* 1989). Spectrum produced by phosphate determination exhibited a maximum absorption around 880 to 890 nm. A characteristic shoulder was seen around 700 to 720 nm (Hori *et al.* 1988). All of the Mo-blue spectra from previously isolated Molybdenum-reducing bacteria showed similar spectra. Based on this knowledge, a hypothesis was developed for this study i.e. molybdenum reduction should proceed via the intermediate phosphomolybdate (Shukor *et al.* 2007). Another basis for the hypothesis was the confirmation of the presence of phosphomolybdate species through spectrophotometric analysis of the resultant absorption spectrum (Sims 1961; Yoshimura *et al.* 1986; Hori *et al.* 1988).

Aside from the demonstration of the unique spectroscopic profile of the blue product, the presence of Mo-blue by itself can visually prove that molybdenum reduction has taken place. Reduced phosphomolybdate or Mo-blue has a fractional oxidation state between 6+ and 5+ (Sidgwick 1951; Kazansky & Fedotov 1980). Other metals, such as mercury and chromate, show no visible color when experiencing reduction through bacterial process (Suzuki *et al.* 1992). Therefore, confirmation of reduction has to be carried out through EPR-Electron Paramagnetic Resonance (Suzuki *et al.* 1992). The presence of an intermediate species is not unique

to Mo-blue production, as this is also reported in bacterial chromate reduction, such as bacteria *Pseudomonas ambigua* (Suzuki *et al.* 1992) and *Shewanella putrefaciens* (now known as *S. oneidensis*) (Myers *et al.* 2000). In these bacterial reduction processes, an intermediate species Cr^{5+} was observed (Myers *et al.* 2000).

Effect of Electron Donor on Molybdate Reduction

Molybdate reduction to Mo-blue was best supported by the sugar glucose. This was followed by sucrose, maltose, l-rhamnose, raffinose, d-mannose, lactose, mucate, cellobiose, d-mannitol, d-adonitol, melibiose, glycerol, d-sorbitol and l-arabinose in descending order (Fig. 5). Mo-blue production by other sugars such as dulcitol, salicin and myo-inositol were found to be not significantly different ($p > 0.05$) from control based on ANOVA with Tukey's test. Previous works demonstrated that Molybdenum-reducing bacteria prefer simple assimilable sugars, such as sucrose, glucose and fructose (Campbell *et al.* 1985; Ghani *et al.* 1993; Rahman *et al.* 2009; Yunus *et al.* 2009; Shukor *et al.* 2009; Ahmad *et al.* 2013; Othman *et al.* 2013; Abo-Shakeer *et al.* 2013). The metabolic pathways glycolysis, Kreb's cycle and electron transport chain were used to convert these sugars to NADH and NADPH. Both reducing equivalents are electron donating substrates for the molybdenum reducing-enzyme (Shukor *et al.* 2014).

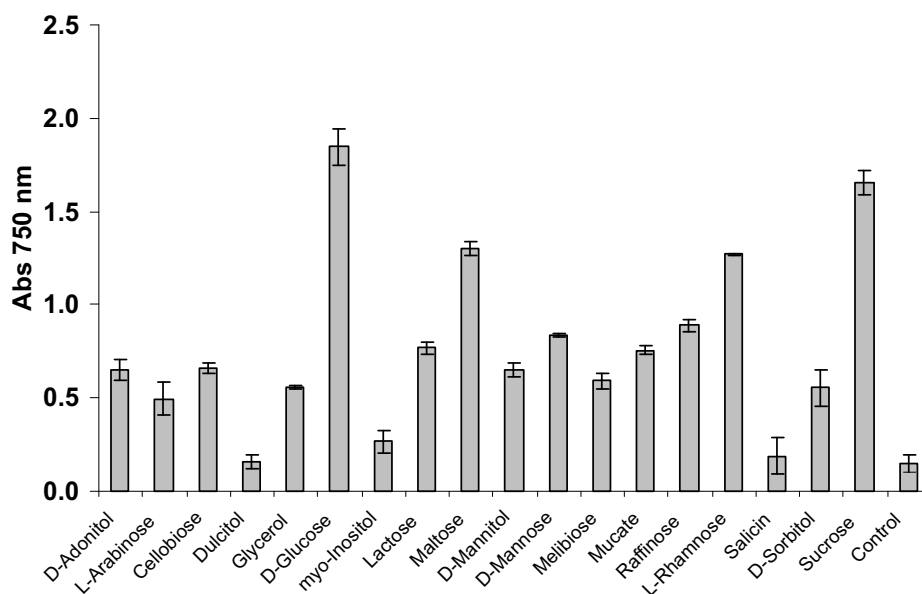


Figure 5 Effect of different electron donor sources (1% w/v) on molybdenum reduction
Note: Error bars represent mean \pm standard deviation (n = 3)

Effect of Phosphate and Molybdate Concentrations to Molybdate Reduction

The optimal concentration of phosphate supporting optimal molybdenum reduction occurred at 5 mM. Concentrations higher than this dramatically ceased production of Mo-blue (Fig. 6). High phosphate concentrations inhibit the stability of phosphomolybdate, of which upon reduction, it is converted to Mo-blue (Glenn & Crane 1956; Sims 1961; Shukor *et al.* 2000). Molybdenum-reducing bacteria isolated previously are also strongly inhibited by

phosphate concentration higher than 5 mM (Shukor & Syed 2010; Lim *et al.* 2012; Ahmad *et al.* 2013; Halmi *et al.* 2013; Othman *et al.* 2013; Abo-Shakeer *et al.* 2013; Khan *et al.* 2014; Shukor *et al.* 2014).

Enterobacter sp. strain Saw-2 can tolerate and reduce molybdenum at sodium molybdate concentrations as high as 60 mM, but the production of Mo-blue was severely inhibited. The optimal concentrations of molybdate which supported the reduction, were between 15 and 30 mM (Fig. 7). The other Molybdenum-reducing

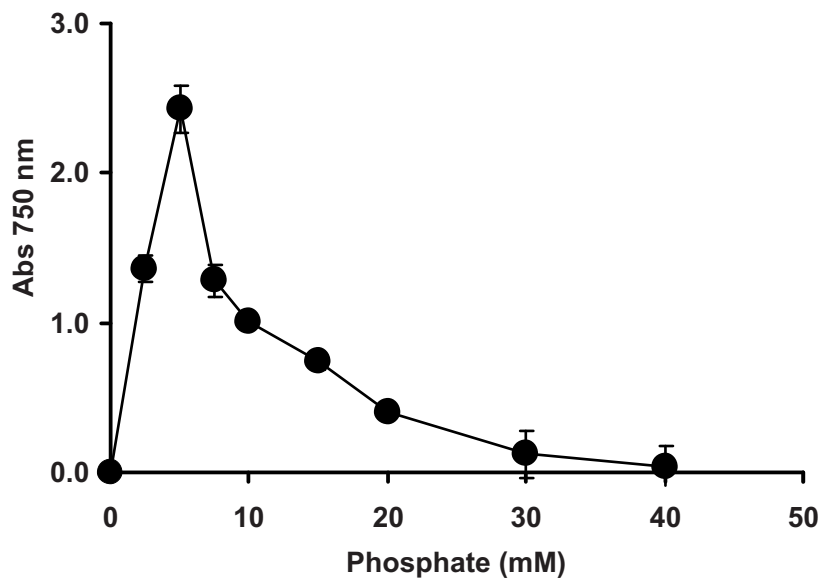


Figure 6 Molybdenum reduction by *Enterobacter* sp. strain Saw-2 at various phosphate concentrations
Note: Error bars represent mean \pm standard deviation (n = 3)

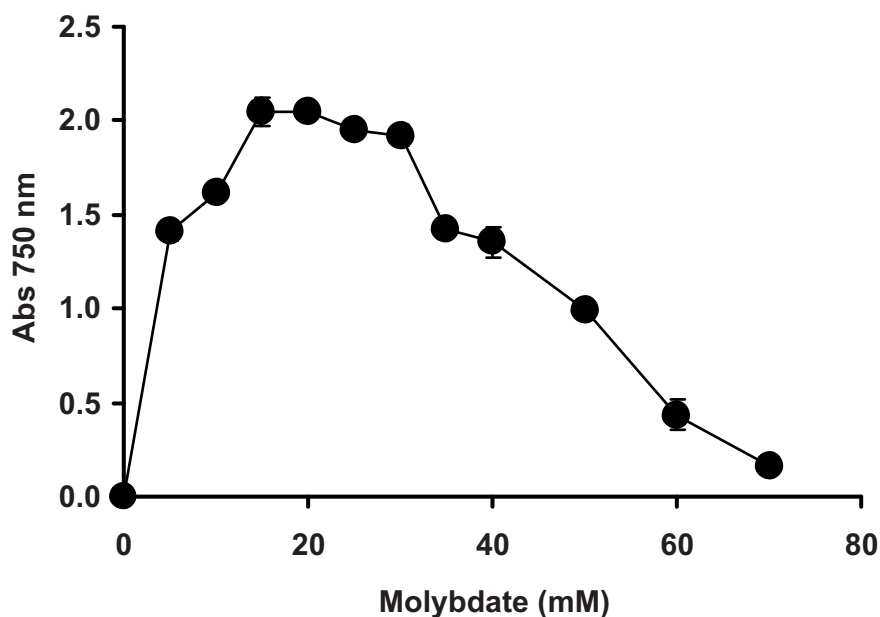


Figure 7 Molybdenum reduction by *Enterobacter* sp. strain Saw-2 at various sodium molybdate concentrations
Note: Error bars represent mean \pm standard deviation (n = 3)

bacteria isolated to date, require optimal molybdate concentration between 10 and 80 mM (Shukor, Rahman *et al.* 2010; Shukor & Syed 2010; Lim *et al.* 2012; Ahmad *et al.* 2013; Halmi *et al.* 2013; Othman *et al.* 2013; Abo-Shakeer *et al.* 2013; Shukor *et al.* 2014). Reduction at these very high concentrations is an advantage for molybdenum bioremediation in an area having high concentration of this metal. For instance, in New Mexico where molybdenum concentration as high as 2,000 ppm or about 20 mM was reported (Jacobs *et al.* 2014).

Effect of Heavy Metals

Bacterial molybdate reduction to Mo-blue was inhibited by other heavy metals, which included cadmium (II), mercury (II), copper (II) and silver (I) at 2 ppm with inhibition levels of 45.5, 36.8, 28.4 and 24.4%, respectively. These inhibition levels were compared to control, which was assigned as having 100% activity (Fig. 8).

Molybdate reduction to Mo-blue in many of the previously isolated Molybdenum-reducing bacteria were inhibited by similar toxic heavy metals (Shukor & Syed 2010; Lim *et al.* 2012; Othman *et al.* 2013; Shukor *et al.* 2014). In hexavalent chromate reduction to the trivalent state by *Bacillus* sp. (Arutchelvan *et al.* 2006) and *Enterobacter cloacae* strain H01 (Rege *et al.* 1997), the

metal ions of mercury and copper were strong inhibitors. The supposed target of these metals was chromate reductase. This enzyme utilized the electron donors NADH and NADPH to convert soluble toxic chromium 6+ to the insoluble less toxic chromium 3+. Mercury binds strongly to sulfhydryl groups. Additionally, mercury also binds, with variable strength, to carboxyl, amide, phosphoryl and amine groups of protein. This is the reason why mercury is one of the most toxic metal ions known to human. Silver also binds to the sulfhydryl group of enzymes. Copper preferentially binds to cysteine, histidine and methionine residues of enzymes (Camakaris *et al.* 1999). The toxicity of these metal ions can be remedied through the addition of chemical additives including phosphate, calcium carbonate, manganese oxide and magnesium hydroxide (Hettiarachchi *et al.* 2000; Deeb & Altalhi 2009).

Phenolics as Carbon Sources for Molybdenum Reduction and Independent Growth

Preliminary screening works on phenolics as carbon sources supporting molybdenum reduction failed to give positive results (Data not shown). However, the bacterium was able to grow on the phenolic compounds (phenol and catechol) (Fig. 9).

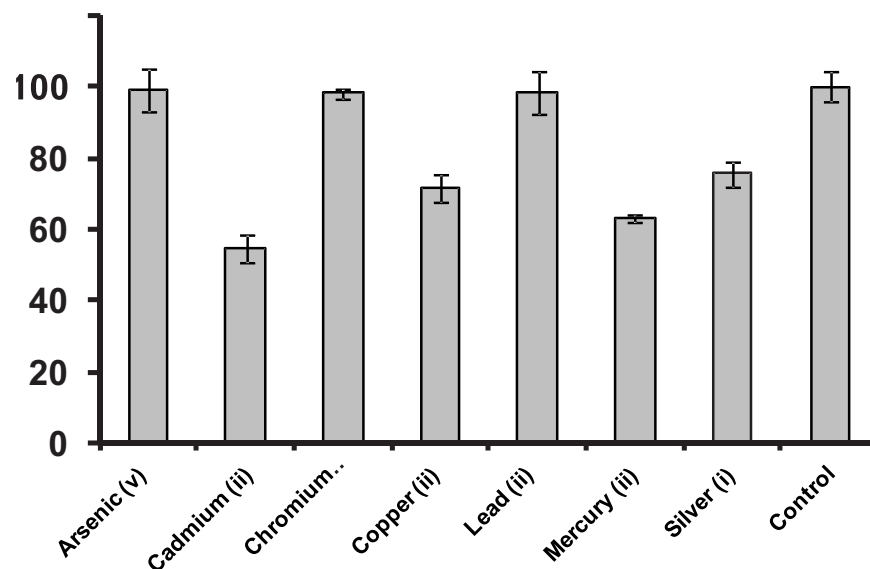


Figure 8 The effect of other heavy metals on Mo-blue production by *Enterobacter* sp. strain Saw-2
Note: Error bars represent mean \pm standard deviation (n = 3)

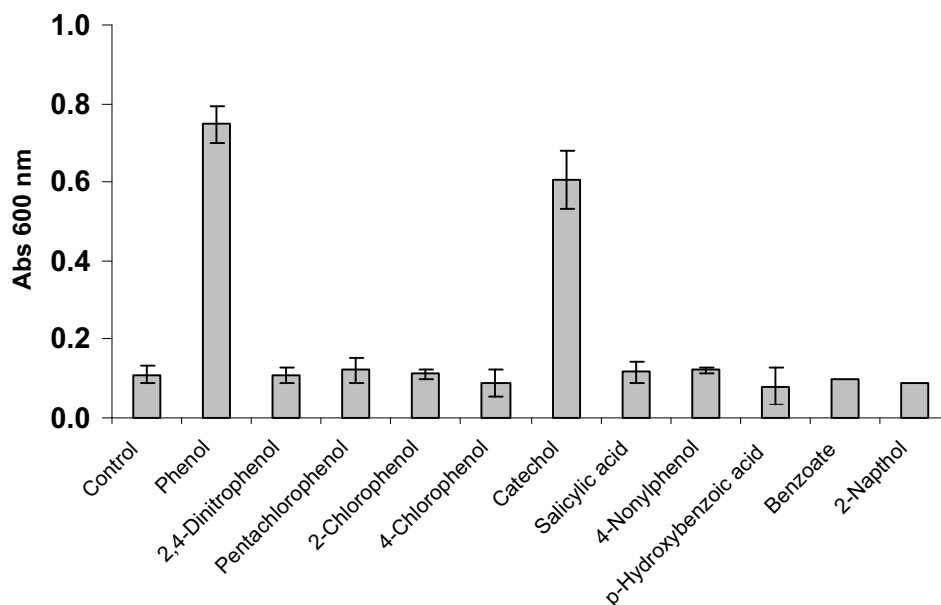


Figure 9 Growth of *Enterobacter* sp. strain Saw-2 on phenolics independent of molybdenum reduction
Note: Error bars represent mean \pm standard deviation (n = 3)

Biodegradation of phenol and phenolic compounds by microorganisms has long been an object of research. Bacteria that could degrade phenol and phenolic compounds include *Pseudomonas* species (Folsom *et al.* 1990; Tomasi *et al.* 1995; Aravindhan *et al.* 2014; Hasan & Jabeen 2015), *Bacillus brevis* (Arutchelvan *et al.* 2006), *Alcaligenes* sp. (Bai *et al.* 2007), *Ochrobactrum* sp. (Kiliç 2009), *Acinetobacter* sp. (Ahmad *et al.* 2011; Yadzir *et al.* 2016) and *Rhodococcus species* (Arif *et al.* 2013).

CONCLUSIONS

Enterobacter sp. strain Saw-2 showed the novel ability to reduce heavy metal molybdenum to Mo-blue. This bacterium can also grow on the phenolic compounds (phenol and catechol). The identity of this bacterium is not completely robust; therefore, molecular identification is needed to further identify this species. This bacterium demonstrated a narrow pH and temperature ranges for optimal reduction. Glucose was the most effective electron donor for optimal molybdenum reduction. The bacterium needed a critical phosphate concentration of 5.0 mM. The optimal molybdate concentrations were between 15 and 30 mM. The absorption spectrum of the Mo-blue generated indicated that it was a reduced phosphomolybdate. Cadmium (II), mercury (II),

copper (II) and silver (I) inhibited the process of molybdenum reduction to Mo-blue. Presently, efforts are ongoing to purify the Molybdenum-reducing enzyme and to characterize phenolics biodegradation studies in greater detail. From bioremediation point of view, this reported bacterium might be an important bio-tool in reducing environmental pollutants like molybdenum.

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