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ISOLATION AND CHARACTERIZATION OF A MOLYBDENUM-REDUCING AND GLYPHOSATE-DEGRADING *Klebsiella oxytoca* STRAIN SAW-5 IN SOILS FROM SARAWAK

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ABSTRACT

Bioremediation of pollutants including heavy metals and xenobiotics is an economic and environmentally friendly process. A novel molybdenum-reducing bacterium with the ability to utilize the pesticide glyphosate as a carbon source is reported. The characterization works were carried out utilizing bacterial resting cells in a microplate format. The bacterium reduces molybdate to Mo-blue optimally between pH 6.3 and 6.8 and at 34^{o}C. Glucose was the best electron donor for supporting molybdate reduction followed by lactose, maltose, melibiose, raffinose, d-mannitol, d-xylose, l-rhamnose, l-arabinose, dulcitol, myo-inositol and glycerol in descending order. Other requirements include a phosphate concentration at 5.0 mM and a molybdate concentration between 20 and 30 mM. The molybdenum blue exhibited an absorption spectrum resembling a reduced phosphomolybdate. Molybdenum reduction was inhibited by mercury, silver, cadmium and copper at 2 ppm by 45.5, 26.0, 18.5 and 16.3%, respectively. Biochemical analysis identified the bacterium as Klebsiella oxytoca strain Saw-5. To conclude, the capacity of this bacterium to reduce molybdenum into a less toxic form and to grow on glyphosate is novel and makes the bacterium an important instrument for bioremediation of these pollutants.

Keyword: bioreduction; glyphosate; *Klebsiella oxytoca;* molybdenum blue; molybdenum-reducing bacterium

INTRODUCTION

Industrialization activities have produced millions of tons of heavy metals and organic pollutants yearly. Glyphosate is the main component or ingredient in a broad range of herbicide formulations used globally for the control of many weeds. Current usage of glyphosate-resistant transgenic crops have resulted in serious soil pollutions since some farmers abuse the amount of glyphosate they applied to these crops (Duke and Powles, 2008). Toxicity associated with glyphosate is often caused by glyphosate and its inert ingredients used to allow the herbicide to adhere better to weeds. Numerous studies have shown that the formulations and surfactant additives are usually more toxic to test species (Barceló and Hennion, 1995; Giesy *et al.*, 2000). Studies on various organisms such as nematode, juvenile frogs, freshwater crustaceans, microalgae and sturgeons showed that they exhibit significant toxicity to these organisms (Achiorno *et al.*, 2008; Bernal *et al.*, 2009; Ermis and Demir, 2009; Deepananda *et al.*, 2011; Filizadeh and Islami, 2011; Bates and Edwards, 2013; Piola *et al.*, 2013). Removal of glyphosate from soils is best carried out by bioremediation often employing glyphosate-degrading bacteria as the low con-

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centration of glyphosate in polluted soils make other form of remediation such as chemical and physical more expensive and unpractical. Examples of glyphosate-degrading bacterium reported include *Enterobacter cloacae* (Kryuchkova *et al.*, 2014), *Ochrobactrum* spp. (Hadi *et al.*, 2013), *Pseudomonas fluorescens* (Zboinska *et al.*, 1992).

Molybdenum is an essential heavy metal that has many uses in industries especially in the steel industry, and as lubricant in the form of molybdenum disulphide. This broad application of molybdenum has led to several cases of water pollution globally including the Tokyo Bay, an agricultural site in Tyrol, Austria, and waters of the Black Sea (Davis, 1991; Neunhäuserer *et al.*, 2001). Terrestrially, it has been reported as a contaminant in sewage sludge pollution (Neunhäuserer *et al.*, 2001). Molybdenum has been demonstrated to inhibit spermatogenesis process and leads to the arrest of embryogenesis in several organisms for instance catfish and mice at concentrations as little as several parts per million (Yamaguchi *et al.*, 2007; Zhang *et al.*, 2013). Additionally, the toxicity of molybdenum to such as sheep and cows at levels of several parts per million has been reported (Kincaid, 1980). The removal of molybdenum through bioremediation especially bioreduction is potentially the most economical approach in the long term (Lim *et al.*, 2014), and several molybdenum-reducing bacteria have been reported (Shukor and Syed, 2010; Ahmad *et al.*, 2013; Masdor *et al.*, 2015). Heavy metals reduction coupled with xenobiotic degradation has been reported (Chirwa and Wang, 2000; Chung *et al*., 2007). For example, two molybdenum-reducing bacteria with the ability to utilize sodium dodecyl sulfate (SDS) as the sole carbon have been isolated (Halmi *et al.*, 2013; Masdor *et al.*, 2015). The use of foreign microbes for bioremediation could cause ecological problems, and thus is not recommended (Walter *et al.*, 2003). Thus, screening of local microorganisms for remediation works, especially with multiple detoxification ability is the best option.

In order to isolate microorganisms with multiple detoxification capacity, a molybdenum-reducing bacterium isolated from contaminated agricultural soil was monitored for its ability to use pesticides as electron donor for reduction or to be able to utilize independently as carbon sources for growth. The Mo-reducing bacterium can successfully utilize the pesticide glyphosate as a carbon source although it cannot be used as an electron donor for molybdenum reduction. The property of this bacterium causes it to become well suited for potential bioremediation works relating to both the heavy metal molybdenum and the pesticide glyphosate as the organic contaminants.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade and purchased from Sigma (St. Louis, MO, USA), Fisher (Malaysia) and Merck (Darmstadt, Germany).

Isolation of Molybdenum-Reducing Bacterium

Soil samples were taken (5 cm deep from topsoil) from the grounds of a contaminated agricultural land in Kuching, Sarawak, Malaysia in January 2010. The land has been periodically sprayed with the glyphosate herbicide (Roundup®) for several years. One gram of the soil sample was first suspended in sterile tap water, and 0.1 mL was transferred to an agar of LPM media (pH 7.0), and further incubated at room temperature for 48 hours. 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) (Yunus *et al.*, 2009). Several white and blue colonies formed after the incubation period. The presence of blue colonies indicates the presence of molybdenum-reducing bacteria. The most intense blue colony was repeatedly streaked on low phosphate media (LPM) agar to purify the bacterial culture. Molybdenum blue (Mo-blue) absorption spectrum was studied by taking out 1.0 mL of the bacterial grown in LPM, but in liquid culture. Aliquot of the culture was centrifuged at 10,000 x g for 10 minutes at room temperature. The Mo-blue produced was scanned from the wavelength 400 to 900 nm using a UV-spectrophotometer (Shimadzu 1201). The low phosphate media was utilized as the baseline correction. Molybdenum reduction in liquid media (at pH 7.0) was also conducted in liquid culture (100 mL) in high phosphate molybdate (HPM) media in a 250 mL culture flask for the preparation of resting cells. The culture was incubated for 48 hours at room

temperature on an orbital shaker (120 rpm). In the HPM media, the phosphate concentration was raised to 100 mM.

Morphological, Physiological and Biochemical Characterization of the Mo-reducing Bacterium

The bacterium was biochemically and phenotypically characterized using standard methods such as colony shape, gram staining, size and colour on nutrient agar plate, motility, oxidase (24 hours), arginine dihydrolase (ADH), esculin hydrolysis, nitrates reduction, ONPG (beta-galactosidase), Voges-Proskauer (VP), lysine decarboxylase (LDC), catalase production (24 h), tartrate (Jordans), ornithine decarboxylase (ODC), gelatin hydrolysis, Methyl red, indole production, hydrogen sulfide (H2S), acetate utilization, deoxyribonuclease, malonate utilization, phenylalanine deaminase, lipase (corn oil), citrate utilization (Simmons), urea hydrolysis, production of acids from various sugars and gas production from glucose were carried out according to the Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). The results were then analysed using the ABIS online system (Costin and Ionut, 2015).

Preparation of Resting Cells for Molybdenum Reduction Characterization

Mo-blue production from this bacterium was characterized further in the microplate format, using the resting cells grown in HPM media above. Characterization studies include the studies of temperature, pH, molybdate and phosphate concentrations were carried as previously described (Shukor and Shukor, 2014). Cells were centrifuged at 15,000 x g for 10 minutes. The bacterial pellets formed were first washed several times and then resuspended in 20 mls of low phosphate media (LPM) minus glucose. The bacterial suspension (180 μL) was transferred into the wells of a sterile microplate. Then sterile glucose (20 μL) was mixed to initiate Mo-blue production. The final concentration of glucose was 1% (w/v). The microplate was sealed (Corning® microplate) and incubated at room temperature. The absorbance at 750 nm was read at defined times in a Microtiter Plate reader (BioRad, Richmond, CA, Model No. 680). Mo-blue production was determined using a specific extinction coefficient at 750 nm of 11.69 mM.-1.cm-1 (Shukor *et al.*, 2003).

The Effect of Heavy Metals on Mo-blue Production

The heavy metals copper, lead, silver, chromium, arsenic, mercury, and cadmium were tested at 2 mg L^{-1}. The heavy metals were sourced from Atomic Absorption Spectrometry standard solutions (Merck KGaA, Darmstadt, Germany) or commercial salts. The amount of Mo-blue production was measured at 750 nm as before.

Pesticides as Sources of Electron Donor and Carbon Sources for Growth

The ability of pesticides such as atrazine, carbaryl, flucythrinate, glyphosate, imidacloprid, carbofuran, coumaphos, diazinon, dicamba, diuron, endosulfan, metolachlor, paraquat, parathion, simazine to support molybdenum reduction as electron donors was tested in the microplate format. Glucose was replaced from the LPM media, and the xenobiotics was added to the final concentration of 200 mg L^{-1}. Atrazine, coumaphos, diazinon, diuron, endosulfan, parathion, flucythrinate and simazine were dissolved in minimal volume of methanol as the carrier solvent. Then 200 μL of the media was added into the microplate wells. The ability of these pesticides to support bacterial growth independent of molybdenum reduction was tested using the microplate format above using the following media at the final concentration of 200 mg L^{-1}. The ingredients of the growth media (LPM) were as follows: $(NH_4)_2.SO_4$ (0.3%), $NaNO_3$ (0.2%), $MgSO_4.7H_2O$ (0.05%), yeast extract (0.01%), NaCl (0.5%), Na_2HPO_4 (0.705% or 50 mM) and 1 mL of trace elements solution. The trace elements solution composition (mg L^{-1}) was as follows: $CaCl_2$ (40), $FeSO_4{\cdot}7H_2O$ (40), $MnSO_4{\cdot}4H_2O$ (40), $ZnSO_4{\cdot}7H_2O$ (20), $CuSO_4{\cdot}5H_2O$ (5), $CoCl_2{\cdot}6H_2O$ (5), $Na_2MoO_4{\cdot}2H_2O$ (5). The media was adjusted to pH 7.0. Then 200 μL of the media was added into the microplate wells and incubated at room temperature for 72 hours. The increase of bacterial growth after an incubation period of 3 days at room temperature was measured at 600 nm using the microplate reader (Bio-Rad 680).

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

Klebsiella oxytoca strain Saw-5 was a short rod-shaped, nonmotile, Gram-negative and facultative anaerobe bacterium. Identification of the bacterium was carried out by comparing the results of cultural, morphological and various biochemical tests (Table 1) and via the ABIS online software (Costin and Ionut, 2015). The software gave three suggestions for the bacterial identity with the highest similarity or homology (>95%) and accuracy (100%) as *K. oxytoca*. However, more work in the future especially molecular identification technique through comparison of the 16srRNA gene is needed to identify this species further. However, for now the bacterium is tentatively identified as *K. oxytoca* strain Saw-5. Previously, two molybdenum-reducing bacterium from this genus; *K. oxytoca* strain Dr.Y14 (Halmi *et al.*, 2013) and *K. oxytoca* strain hkeem (Lim *et al.*, 2012) have been isolated.

A microplate format was utilized that allows a high throughput method. The method has the potential to speed up characterization works and potentially obtaining more data compared to the normal shake-flask method (Iyamu *et al.*, 2008; Shukor and Shukor, 2014). The use of resting cells under static conditions in studying characteristics of molybdenum-reducing bacterium was initiated in *Enterobacter cloacae* strain 48 (Ghani *et al.*, 1993). The advantage of this approach is molybdenum reduction proceed best under low oxygen environment of between 0 and 10% of environmental oxygen or EO (Ghani *et al.*, 1993). In a microplate or microtiter format the oxygen concentration is between 0 and 10% of environmental oxygen or EO or more commonly known as static conditions. This value is significantly lower than growth under fully aerobic conditions at approximately 20% EO.

Table 1. Biochemical tests for *Klebsiella oxytoca* strain Saw-5

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

Remarks: + = positive result; − = negative result; d = indeterminate result

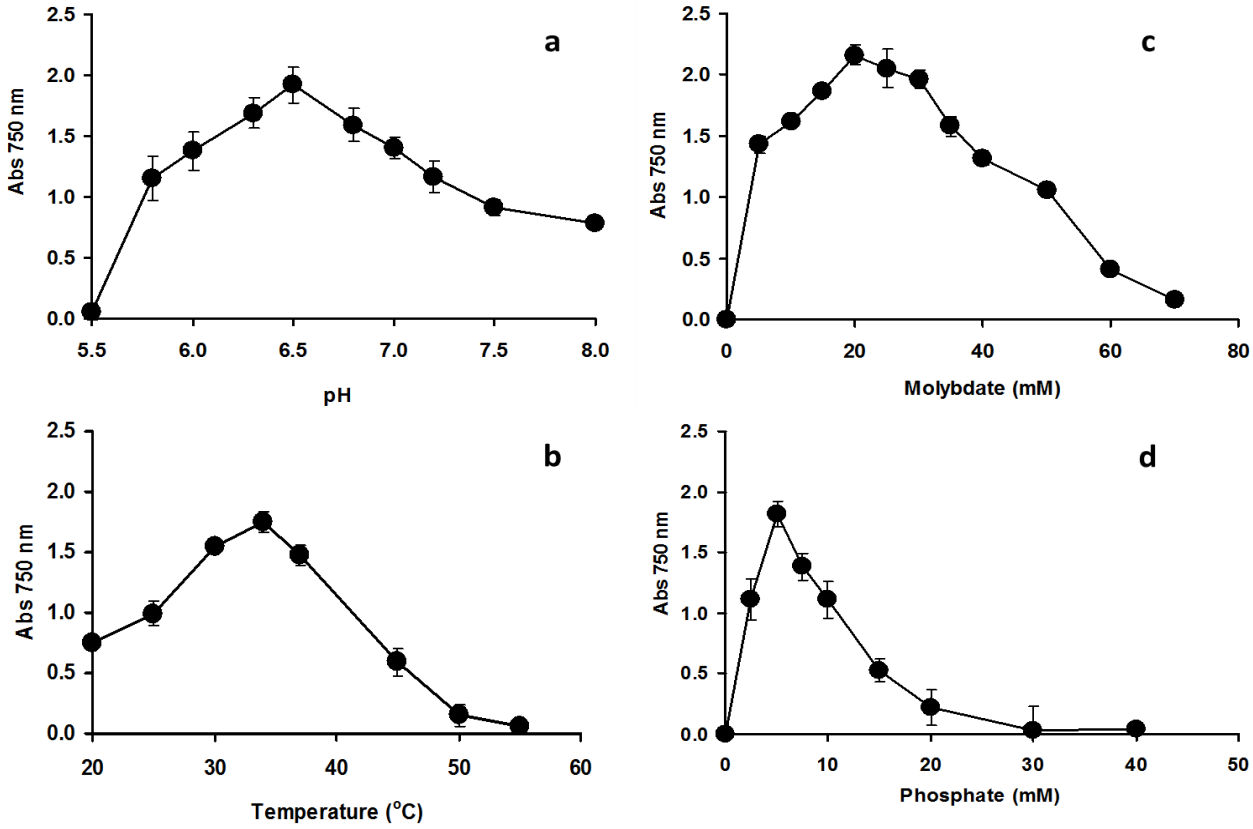

Figure 1. Effect of pH (a), temperature (b), molybdate concentration (c), and phosphate concentration (d) on molybdenum reduction by *Klebsiella oxytoca* strain Saw-5. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 48 hours. Error bars represent mean ± standard deviation (n=3)

The use of static conditions would mimic bioremediation environment especially in aquatic bodies or soils, of which the EO level is much lower than 20% EO. Under this conditions, other electron acceptors such as nitrate or metal ions such as manganese or iron would be used as an alternative (Haley *et al.*, 2012). Resting cells have been used in studying heavy metals reduction such as in selenate (Losi and Jr, 1997), vanadate (Carpentier *et al.*, 2005), chromate (Llovera *et al.*, 1993), reductions and xenobiotics biodegradation such as phenol (Sedighi and Vahabzadeh, 2014), amides (Raj *et al.*, 2010), diesel (Auffret *et al.*, 2015), SDS (Chaturvedi and Kumar, 2011), and pentachlorophenol (Steiert *et al.*, 1987).

Molybdenum Absorbance Spectrum

The scanning absorption spectrum of the molybdenum blue generated by *K. oxytoca* strain Saw-5 displayed 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 (Figure 2a). The identification of the Mo-blue generated is not simply figured out because it is complex in structure and it has numerous species (Shukor *et al.*, 2007). Briefly molybdenum blue is a reduced product of isopolymolybdate and heteropolymolybdate. Campbell *et al.* (1985) were the first to indicate that the Mo-blue observed from *E. coli* K12 is a reduced form of phosphomolybdate, but they did not suggest a plausible mechanism for its formation. Formation of isopoly Mo-blue from molybdate is extremely hard using biological-based reducing agents because the conversion calls for strong reducing agents and under acidic circumstances. The formation of heteropoly Mo-blue by biologically-based reducing agents for example ascorbic acids or enzymatic reduction is much more possible as observed in

the phosphate determination method utilizing ascorbic acid (Hori *et al.*, 1988). It was hypothesized that the microbial molybdate reduction in media that contains molybdate and phosphate need to commence through the phosphomolybdate intermediate, and the transformation from molybdate to this structure takes place as a result of reduction of pH in the course of bacterial growth. To be precise, the spectrum displayed should demonstrate a maximum absorption in between 860 and 870 nm, and a shoulder at about 700 nm. The Mo-blue spectrum from the PDM generally demonstrated a maximum absorption around 880 to 890 nm, and a shoulder around 700 to 720 nm (Shukor *et al.*, 2010a). It was demonstrated beforehand that all of the molybdenum blue spectra obtained from other molybdenum-reducing bacteria follow this prerequisite (Shukor *et al.*, 2007). Here, the results from the absorption spectra clearly imply a similar spectrum, and hence provide some evidence for the hypothesis. Although a detail identification of the actual phosphomolybdate species needs to be carried out using nuclear magnetic resonance (n.m.r) and electron spin resonance (e.s.r.), spectrophotometric characterization of the heteropolymolybdate species through analyzing the scanning spectroscopic profile is a less cumbersome and accepted method (Yoshimura *et al.*, 1986). Although the maximum absorption wavelength for Mo-blue was 865 nm, measurement at 750 nm, even though was about 30% lower, was enough for routine monitoring of molybdenum blue production as the intensity obtained was much higher than cellular absorption at 600-620 nm (Shukor and Shukor, 2014). Previous monitoring of Mo-blue production uses several wavelengths such as 710 nm (Ghani *et al.*, 1993) and 820 nm (Campbell *et al.*, 1985).

Mo-blue Production as A Function of pH and Temperature

Klebsiella oxytoca strain Saw-5 was incubated at different pHs of between 5.5 and 8.0 using 20 mM Bis-Tris and Tris.Cl buffers. Analysis by ANOVA showed that the optimum pH for reduction was observed between pH 6.3 and 6.8. It was also observed that inhibition of reduction was dramatic at pH lower than 5.0 (Figure 1a). The effect of temperature (Figure 1b) showed that the optimum temperature supporting molybdenum reduction was at 34°C with temperatures higher than 34ºC were strongly inhibitory to molybdenum reduction.

Temperature and pH both play important functions in the process of molybdenum reduction as the process is enzyme mediated. Both parameters have an effect on the protein folding and enzyme activity leading to the inhibition of the molybdenum reduction process. The optimum temperature observed in this work is an advantage for bioremediation works in a tropical country like Malaysia, which exhibit average yearly temperature of between 25 and 35ºC (Shukor *et al.*, 2008a). Therefore, *K. oxytoca* strain Saw-5 is a potential candidate for soil bioremediation of sites containing elevated levels of molybdenum either locally or in other tropical countries. The optimum temperature observed in this work falls within the optimal temperature range reported in the majority of the molybdenum reducers isolated to date, which exhibit optimal temperature of between 25 and 37ºC (Shukor and Syed, 2010; Lim *et al.*, 2012; Abo-Shakeer *et al.*, 2013; Othman *et al.*, 2013; Halmi *et al.*, 2013; Khan *et al.*, 2014; Shukor *et al.*, 2014) with the only psychrotolerant reducer isolated from Antartica showing an optimal temperature supporting reduction of between 15 and 20 ºC (Ahmad *et al.*, 2013).

The optimal pH range exhibited by *K. oxytoca* strain Saw-5 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 (Shukor and Syed, 2010; Lim *et al.*, 2012; Abo-Shakeer *et al.*, 2013; Ahmad *et al.*, 2013; Othman *et al.*, 2013; Halmi *et al.*, 2013; Khan *et al.*, 2014; Shukor *et al.*, 2014). 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 (Shukor *et al.*, 2007).

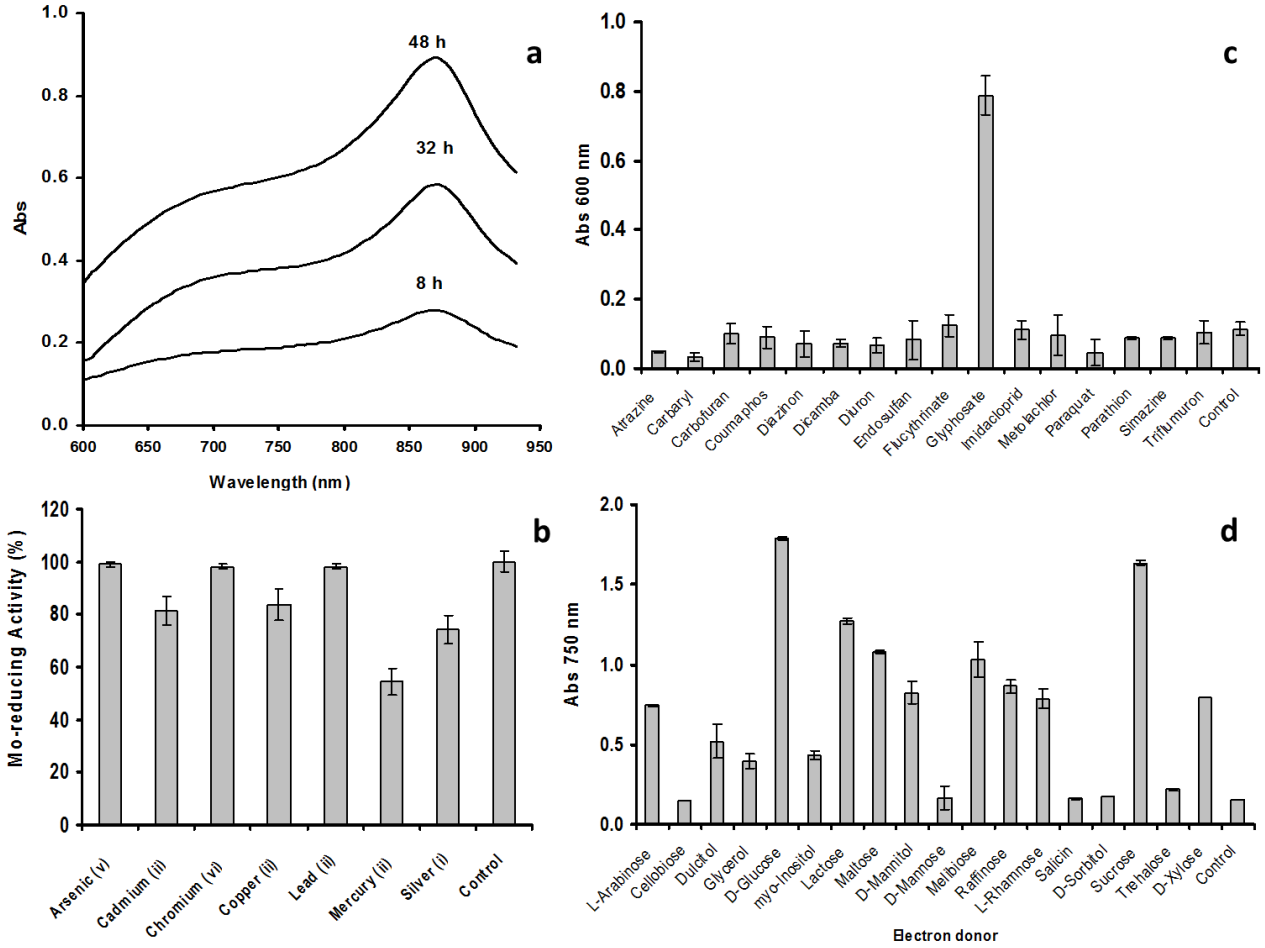

Figure 2. (a) Scanning absorption spectrum of Mo-blue from *Klebsiella oxytoca* strain Saw-5 at different time intervals; (b) Mo-blue production under various heavy metals; (c) Growth of *K. oxytoca* strain Saw-5 on pesticides; (d) Effect of different electron donor sources (1% w/v) on molybdenum reduction. *Klebsiella oxytoca* strain Saw-5 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).

Mo-blue Production Using Various Electron Donors

Mo-blue production was optimum using glucose as the electron donor. This is followed by lactose, maltose, melibiose, raffinose, d-mannitol, d-xylose, l-rhamnose, l-arabinose, dulcitol, myo-inositol and glycerol in descending order (Figure 2b). Several of the molybdenum-reducing bacteria previously isolated such as *Pseudomonas* sp. strain DRY2 (Shukor *et al.*, 2010a), *Pseudomonas* sp. strain DRY1 (Ahmad *et al.*, 2013), *Bacillus pumilus* strain Ibna (Abo-Shakeer *et al.*, 2013) and *Bacillus* sp. strain A.rzi (Othman *et al.*, 2013), *Escherichia coli* K12 (Campbell *et al.*, 1985), *Serratia* sp. strain Dr.Y5 (Rahman *et al.*, 2009), *Enterobacter* sp. strain Dr.Y13 (Shukor *et al.*, 2009a), *Acinetobacter calcoaceticus* strain Dr.Y12 (Shukor *et al.*, 2010b), prefer glucose as the electron donor, while *Enterobacter cloacae* strain 48 (Ghani *et al.*, 1993), *Serratia* sp. strain Dr.Y5 (Rahman *et al.*, 2009), *S. marcescens* strain Dr.Y9 (Yunus *et al.*, 2009) and *S. marcescens* strain DRY6 (Shukor *et al.*, 2008a) prefer sucrose as the best electron donor. Only *K. oxytoca* strain hkeem prefers fructose (Lim *et al.*, 2012). These carbohydrates could produce electron-donating substrates such as NADH and NADPH using generic metabolic pathways including glycolysis, Kreb's cycle, and the electron transport chain. NADH and NADPH are

substrates for the molybdenum reducing-enzyme (Shukor *et al.*, 2008b; Shukor *et al.*, 2014).

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 (Shukor *et al.*, 2008a; Shukor *et al.*, 2009a; Yunus *et al.*, 2009; Shukor *et al.*, 2009b; Shukor *et al.*, 2010a; Shukor *et al.*, 2010b; Lim *et al.*, 2012; Ahmad *et al.*, 2013; Othman *et al.*, 2013; Shukor *et al.*, 2014). The optimum concentration of phosphate occurred at 5 mM with higher concentrations were strongly inhibitory to reduction (Figure 1d). High phosphate was suggested to inhibit phosphomolybdate stability as the complex requires acidic conditions. In addition, the phosphomolybdate complex itself is unstable in the presence of high phosphate through an unknown mechanism (Glenn and Crane, 1956; Sims, 1961; Shukor *et al.*, 2000). All of the molybdenum-reducing bacterium isolated so far requires phosphate concentration not higher than 5 mM for optimal reduction (Shukor and Syed, 2010; Lim *et al.*, 2012; Abo-Shakeer *et al.*, 2013; Ahmad *et al.*, 2013; Othman *et al.*, 2013; Halmi *et al.*, 2013; Khan *et al.*, 2014; Shukor *et al.*, 2014). 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 (Figure 2b). 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 (Shukor *et al.*, 2010a), whilst the highest molybdenum required for optimal reduction was 80 mM in *E. coli* K12 (Campbell *et al.*, 1985) and *K. oxytoca* strain hkeem (Lim *et al.*, 2012). Other Mo-reducing bacteria such as EC48 (Ghani *et al.*, 1993), *S. marcescens* strain Dr.Y6 (Shukor *et al.*, 2008a), *S. marcescens*. Dr.Y9 (Yunus *et al.*, 2009), *Pseudomonas* sp. strain Dr.Y2 (Shukor *et al.*, 2010a), *Serratia* sp. strain Dr.Y5 (Rahman *et al.*, 2009), *Enterobacter* sp. strain Dr.Y13 (Shukor *et al.*, 2009a) and *Acinetobacter calcoaceticus* (Shukor *et al.*, 2010b) 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 (Runnells *et al.*, 1976).

Effect of Heavy Metals

Molybdenum reduction was inhibited by the heavy metals mercury (ii), silver (i), cadmium (ii) and copper (ii) at 2 mg L^{-1} by 45.5, 26.0, 18.5 and 16.3%, respectively (Figure 2b). The presence of these metal ions is a major problem in bioremediation. Therefore, the screening and isolation of molybdenum-reducing bacteria with as many metal resistance capability is important. Heavy metals for example cadmium, silver, mercury, and copper usually target the sulfhydryl group of enzymes (Sugiura and Hirayama, 1976; Sabullah *et al.*, 2014; Sabullah *et al.*, 2015), while chromate is known to inhibit the enzyme glucose oxidase (Zeng *et al.*, 2004). Binding of these heavy metals leads to the inactivation of the metal-reducing capacity of the enzyme(s) responsible for molybdenum reduction.

Pesticides as Electron Donors for Molybdenum Reduction and Independent Growth

The ability of the pesticides to support molybdenum reduction was explored. None of the pesticides could support molybdenum reduction, however, the bacterium could grow on glyphosate (Figure 2d). Numerous glyphosate-degrading bacteria has been isolated and include *Enterobacter cloacae* (Kryuchkova *et al.*, 2014), *Ochrobactrum* spp. (Hadi *et al.*, 2013), *Pseudomonas fluorescens* (Zboinska *et al.*, 1992), *Agrobacterium radiobacter* (McAuliffe *et al.*, 1990), *Pseudomonas* sp. (Bazot and Lebeau, 2008), *P. fluorescens*, *Acetobacter* sp. (Moneke *et al.*, 2010), and bacterial genera such as *Escherichia, Klebsiella, Arthrobacter, Bacillus,* and *Rhizobium* (Schowanek and Verstraete, 1990; Kononova and Nesmeyanova, 2002).

CONCLUSION

A local isolate of Mo-reducing bacterium with the novel ability to use the glyphosate as a source of carbon for growth has been isolated. The screening and characterization works have been carried out using a microplate format. The bacterium was tentatively identified as *Klebsiella*

oxytoca strain Saw-5 based on biochemical methods. However, works are currently being carried out to sequence the 16s rRNA gene from this bacterium followed up by molecular phylogenetic analysis as a further identification method. This is the first report of a molybdenum-reducing bacterium with the capacity to grow on glyphosate. Glucose, an easily assimilable source was the best electron donor. Other requirements include a strict phosphate concentration at 5.0 mM. The absorption spectrum of the molybdenum blue produced indicates that it is a reduced phosphomolybdate. Molybdenum reduction was inhibited by heavy metals similar to previously isolated molybdenum-reducing bacteria. As this bacterium is able to detoxify the heavy metal molybdenum and degrade the pesticide glyphosate, these novel properties will make the bacterium very useful in bioremediation. Presently, the molybdenum-reducing enzyme from this bacterium is being purified and the pesticide-degrading ability is being charac-terized in greater detail.

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