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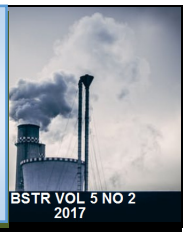

Isolation and Characterisation of a Mo-reducing Bacterium from Malaysian Soil

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ABSTRACT

The issue of heavy metal contamination and toxic xenobiotics has become a rapid global concern. This has ensured that the bioremediation of these toxicants, which are being carried out using novel microbes. A bacterium with the ability to reduce molybdenum has been isolated from contaminated soils and identified as *Serratia marcescens* strain DR.Y10. The bacterium reduced molybdenum (sodium molybdate) to molybdenum blue (Mo-blue) optimally at pHs of between 6.0 and 6.5 and temperatures between 30°C and 37°C. Glucose was the best electron donor for supporting molybdate reduction followed by sucrose, adonitol, mannose, maltose, mannitol glycerol, salicin, myo-inositol, sorbitol and trehalose in descending order. Other requirements include a phosphate concentration of 5 mM and a molybdate concentration of between 10 and 30 mM. The absorption spectrum of the Mo-blue produced was similar to the previously isolated Mo-reducing bacterium and closely resembles a reduced phosphomolybdate. Molybdenum reduction was inhibited by Hg (ii), Ag (i), Cu (ii), and Cr (vi) at 78.9, 69.2, 59.5 and 40.1%, respectively. We also screen for the ability of the bacterium to use various organic xenobiotics such as phenol, acrylamide, nicotinamide, acetamide, iodoacetamide, propionamide, acetamide, sodium dodecyl sulfate (SDS) and diesel as electron donor sources for aiding reduction. The bacterium was also able to grow using amides such as acrylamide, propionamide and acetamide without molybdenum reduction. The unique ability of the bacterium to detoxify many toxicants is much in demand, making this bacterium a vital means of bioremediation.

INTRODUCTION

Since the early days of industrialization revolution, organic and inorganic pollutions are the silent threats that have affected the quality of life. The contamination of water bodies and soils by a multitude of pollutants such as xenobiotics and heavy metals need to be rapidly removed and remediated [1]. Molybdenum is obtained from minerals such as molybdenite (MoS_2), wulfenite ($PbMoO_4$), ferrimolybdite ($FeMoO_3.xH_2O$), and jordisite (amorphous MoS_2). The largest molybdenum producing country is America, producing nearly half of the world production of molybdenum [2]. Molybdenum has also been known to cause significant sewage sludge pollution that poses a health hazard [3]. Several reports have been made by researchers regarding molybdenum toxicity in inhibiting spermatogenesis and arresting embryogenesis in organisms such as catfish and mice at levels as low as several parts per million. Cows are the best example of the most affected ruminants, with scouring and deaths observed at levels as low as several parts per million [4] [5]. At this level, the toxicity of molybdenum is at par with other heavy metals such as chromium and lead [6].

Acrylamide, nicotinamide, acetamide, iodoacetamide, propionamide, acetamide, sodium dodecyl sulfate (SDS),

phenol and diesel are major manmade chemicals (xenobiotics) global pollutants besides heavy metals [7]. These chemicals are being hugely produced every year with a significant proportion of them causing pollutions. Other methods such as physical or chemical methods are effective to remove heavy metals and organic pollutants at high levels, but not a low levels often observed in many polluted sites. Bioremediation would be the technology of choice as it is the most economical and effective approach in the long term especially at low concentrations [8].

Acrylamide is a xenobiotic. It is a toxic compound having been related to exhibit neurotoxicity in humans alongside carcinogenicity and reproductive toxicity in animal models [9]. It is a monomer of the polyacrylamide polymer. The acrylonitrile-acrylamide industries are known sources acrylamide pollution with levels as high as 1 g/L have been reported [10]. The extent usage of acrylamide in treating drinking water has been suspected of being a probable source of acrylamide pollution. In the Sarawak state along, the Kuching Water Board uses nearly a ton of polyacrylamide every year [11].

However, there are some microbes with the ability to degrade a range of xenobiotics. Due to the versatility of these microbes, they have been much sought after for polluted sites where contaminants are viewed as the norm [12]. There has been a report on the reduction of heavy metals coupled with degradation of xenobiotics [13,14]. A study done previously showed that a bacterium with the ability of molybdenum-reduction is able to grow on sodium dodecyl sulfate (SDS) as its sole carbon even though SDS was shown not to support molybdenum reduction [15].

The ability of molybdenum reducing bacterium isolated from contaminated soil to use xenobiotics as an electron donor for reduction process or to utilize these xenobiotics independently as a carbon source for growth was screened. Static growth or conditions are simulating most bioremediation conditions where the environmental oxygen or EO level is less than ~20% were utilized.
This condition is easily achieved in a microplate environment where oxygen concentration of between 0 and 10% environmental oxygen, EO which is lower than under aerobic conditions normally at approximately 20% EO [16]. The characteristics of the bacterium reported in this work, which is having the capacity to grow on acrylamide and detoxify heavy metal would make it suitable for future bioremediation works involving both heavy metal molybdenum contamination and amides as organic contaminants.

MATERIALS AND METHODS

Molybdenum-reducing bacterium isolation

Samples of soils were collected from Bukit Gantang in the State of Perak, Malaysia in January 2005. The samples were taken from the topsoil of the location, 5 cm from the surface. Each soil sample (one gram) was suspended in sterile tap water. An aliquot of 0.1 mL of the soil suspension was spread onto a low phosphate agar media with a pH 7.0. Incubation of the plates was carried out at room temperature for 24 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) [17]. Blue colonies were formed. This indicated that the molybdenum-reducing bacteria have reduced molybdenum. The colony with the most intense blue colour was identified and isolated.

This colony was then restreaked on a low phosphate media (LPM) thus obtaining a 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 × 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 baseline correction was low phosphate media.

16S rRNA gene sequencing

The 16S rRNA forward primer (First Base Sdn Bhd., Malaysia) used was 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer used was 5'-AAGGAGGTGATCCAGCCGCA-3'. The 16s rRNA ribosomal gene sequence was deposited in the GenBank database under the accession number DQ226211.

Identification of bacterium

Identification of the bacterium was carried through molecular phylogenetic analysis. Multiple alignments of closely matched 16S rRNA gene sequences retrieved from GenBank were aligned using the program Clustal W [18]. The PHYLIP format was selected as the output option. Finally, the TreeView program was utilized to view the tree [19].

Preparation of resting cells for molybdenum reduction characterization

Resting cells in a microplate or microtiter format as previously developed by Shukor et al (2012),[11] were used to carry out characterization works on molybdenum reduction to Mo-blue such as the effects of pH, temperature, phosphate and molybdate concentrations. Cells harvesting was carried out by centrifugation process at 15,000 × g for 10 minutes. The phosphate concentration was done by allowing the cells from a 1 L overnight culture to grow in High Phosphate media (HPM) at room temperature on an orbital shaker (150 rpm) with the, difference between the LPM and HPM, which was at 100 mM for the HPM. Molybdate reduction was found to strongly inhibit high concentrations [21,22].

The pellet was washed several times to remove residual phosphate and resuspended in 20 mL of low phosphate media (LPM) minus glucose to an absorbance at 600 nm of about 1.00. 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 [23].

Effect of pH, temperature electron donor, phosphate and molybdate concentrations on molybdate reduction.

Different pH ranging from 5.5 to 8.0 using Bis-Tris and Tris.HCl buffers (20 mM); temperature from 20 to 60 °C; 20

electron donor (carbon sources) such as adonitol, arabinose, cellobiose, dulcitol, glycerol, glucose, myo-inositol, lactose, maltose, mannitol, mannose, melibiose, mucate, raffinose, rhamnose, salicin, d-sorbitol, sucrose, trehalose and xylose; different phosphate ranging from 0 to 40 mM and molybdate from 10 to 70 mM we used. The bacterium was incubated with heavy metals in the microplate format at various concentrations. The plate was incubated for 24 hours at 30 ºC. The amount of Mo-blue production was measured at 750 nm as before.

Effect of heavy metals on molybdenum reduction

Preparation of seven types of heavy metals specifically leads (ii), arsenic (v), copper (ii), mercury (ii), silver (i), chromium (vi) and cadmium (ii) were made either from commercial salts or standard solutions of Atomic Absorption Spectrometry (MERCK). Incubation of the bacterium with the heavy metals occurred in the microplate format at various concentrations for 24 hours at the temperature of 30ºC. The quantity of Mo-blue formed was then measured at 750 nm as before.

Screening for bacterial growth on xenobiotics independent of molybdenum reduction

Xenobiotics namely phenol, acrylamide, nicotinamide, acetamide, iodoacetamide, propionamide, Sodium Dodecyl Sulfate (SDS) and diesel were tested for the ability to support bacterial growth independent of molybdenum reduction. Tests were conducted using the microplate format above while using the following media in addition to the final concentration of the xenobiotics to be 500 mg/L. The growth media was excluded to contain a high concentration of molybdenum for fear of the damaging effect to the growth on xenobiotics.

The ingredients of the growth media 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 [24] with composition (mg/L) 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 pH of the media was modified to a pH 7.0. Initially, diesel was added to the final concentration of 0.5 g/L in 10 mL media and then sonicated for 5 minutes. Next, 200 µL of the media was added into the microplate wells followed by incubation at room temperature for 72 hours. After an incubation period of 3 days at room temperature, the amount of increase of bacterial growth was measured at 600 nm using the microplate reader (Bio-Rad 680).

Statistical analysis

Data analyses were carried out using GraphPad Prism version 5.0 available from www.graphpad.com. A one-way analysis of variance with post hoc analysis by the Tukey's test or a Student's t-test was utilised for between groups comparison. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Identification of molybdenum reducing bacterium

Serratia marcescens strain DR.Y10 was a short rod-shaped, motile, Gram-negative and facultative anaerobe bacterium. The colonies were between 1 to 3 mm in diameter, shiny, cream white, smooth and circular. *S. marcescens* strain DR.Y10 belongs to the group Serratia according to the blast results from NCBI. Phylogenetic analysis (**Fig. 1**) showed a moderate bootstrap value (45.2%) to *S. marcescens* isolate CP01[4]CU. Thus, this bacterium is tentatively identified as *S. marcescens* strain DR.Y10.

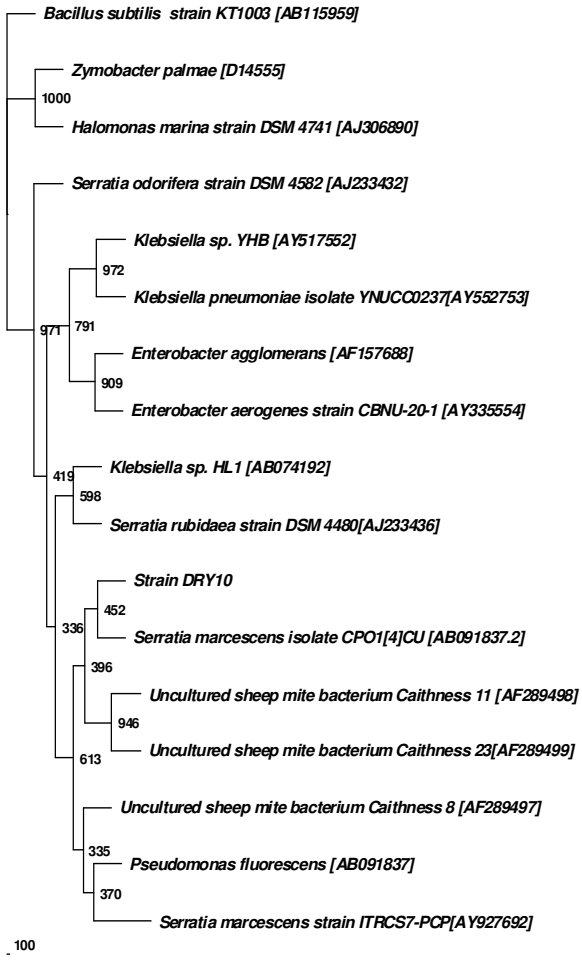

Fig. 1. A phylogram of a neighbour-joining method showing the genetic relationship between *Serratia marcescens* strain DR.Y10 and another bacterium. The outgroup was *Bacillus subtilis*. Species names were followed by the 16S rRNA accession number. The internal labels at the branching points refer to bootstrap value.

In this work, we have isolated new molybdenum reducing bacterium from contaminated soil. Phylogenetic analysis shows that this bacterium belongs to the genus *Serratia* sp. Previous works have shown that the *Serratia* genus is a dominant genus for molybdenum reduction with species isolated so far includes *Serratia* sp. strain DR.Y5 [25], *S. marcescens*. DR.Y6 [26] and *S. marcescens* DR.Y9 [17]. We employed a rapid and simple high throughput method involving microplate format to speed up characterization works and obtaining more data than the normal shake-flask approach [27]. The use of resting cells under static conditions to characterize molybdenum reduction in bacterium was utilised [28]. Resting cells have been used in studying heavy metals reduction such as in selenate [29] and SDS biodegradation [30].

Molybdenum absorbance spectrum

The Mo-blue produced by *S. marcescens* strain DR.Y10 gave off an absorption spectrum which exhibited a unique property. It displayed a shoulder at approximately 700 nm and a maximum peak of between 860 and 870 nm, with a median at 865 nm (**Fig. 2**). The identity of the Mo-blue is not easily determined as the structure is complex. In addition, the

compound can exist in many varieties [31]. The absorption spectrum of the Mo-blue from this bacterium, if it goes through this mechanism, should show a spectrum closely resembling the phosphate determination method with maximum absorption in between 860 and 870 nm and a shoulder at approximately 700 nm. The Mo-blue spectrum from the phosphate determination method normally showed a maximum absorption around 880 to 890 nm and a shoulder around 700 to 720 nm [32]. We have shown previously that the entire Mo-blue spectra from other bacteria obey this requirement [31].

Based on the result from the absorption spectrum, this work shows a similar spectrum, thus providing evidence for the hypothesis. E.S.R. and N.M.R. are methods that can be used to correctly identify the phosphomolybdate species. However, spectrophotometric characterization by analyzing the heteropolymolybdate species scanning spectroscopic profile is easier to handle and caught most of the researchers' attention. There are several wavelengths that were used in the previous monitoring of Mo-blue production such as 710 nm [28] and 820 nm [34]. Although the maximum absorption wavelength for Mo-blue was approximately 30% lower than 865 nm, which is at 750 nm, it was sufficient to routinely monitor the Mo-blue production as the intensity obtained was higher than cellular absorption at 600-620 nm [20].

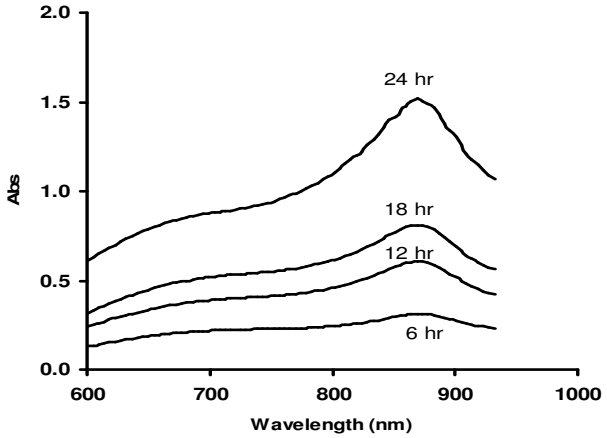

Fig. 2. Scanning absorption spectrum of Mo-blue from *Serratia marcescens* strain DR.Y10 at different time intervals.

Effect of temperature and pH on molybdate reduction

The effect of temperature (**Fig. 4**) was observed over a wide range of temperature (20°C 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 higher than 37°C were strongly inhibitory to Mo-blue production from *S. marcescens* strain DR.Y10. *S. marcescens* strain DR.Y10 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**).

Molybdenum reduction is a process that is mediated by enzymes, of which both temperatures and pH play imperative roles in. Both parameters can affect protein folding and enzyme activity which can cause the inhibition of molybdenum reduction. In a tropical country like Malaysia where the average yearly temperature ranges from 25 to 35°C can provide optimum conditions for bioremediation [26]. Hence, *Serratia marcescens* strain DR.Y10 is a promising candidate for soil bioremediations of molybdenum both locally and in other tropical countries. The majority of reducers were isolated from tropical soils thus exhibiting an optimal temperature ranging between 25 and 37°C [8,20]. The only psychrotolerant reducer which was isolated from Antarctica showed an optimal temperature of between 15 and 20°C which supports reduction [35].

The optimal pH range exhibited by *S. marcescens* strain DR.Y10 reflects the property of the bacterium as a neutrophile in supporting molybdenum reduction, due to the characteristics of neutrophils that are able to grow between pH 5.5 and 8.0. The optimal pH reduction regarding molybdenum reduction in bacteria has been observed, which is slightly acidic with optimal pHs ranging from pH 5.0 to 7.0 [25,36]. The optimal reduction will occur if enzyme activity and substrate stability are a balance which suggested that acidic pH plays an important role in the formation and stability of phosphomolybdate before it is being reduced to Mo-blue [31].

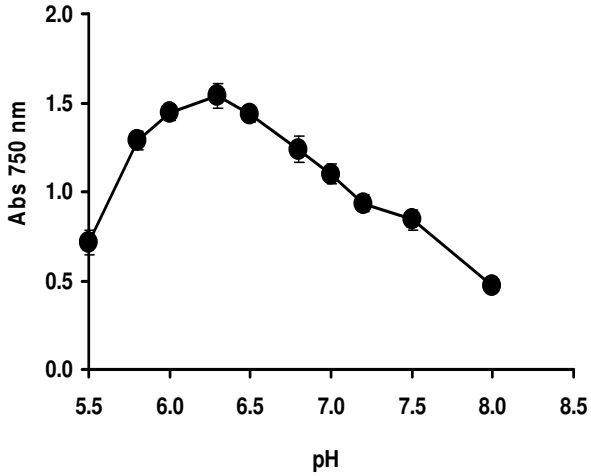

Fig. 3. Effect of pH on molybdenum reduction by *Serratia marcescens* strain DR.Y10. Error bars represent mean ± standard deviation (n=3).

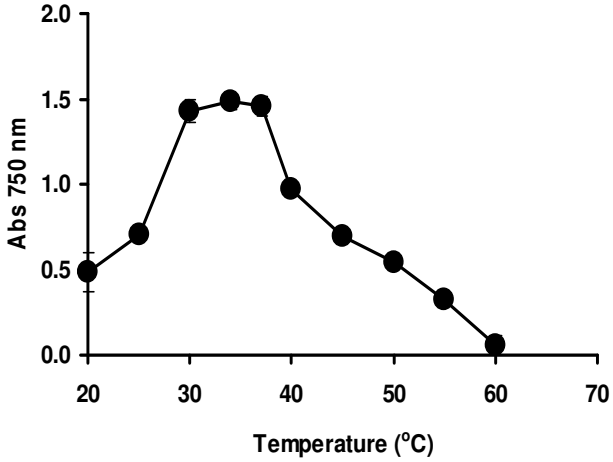

Fig. 4. Effect of temperature on molybdenum reduction by *Serratia marcescens* strain DR.Y10. Error bars represent mean ± standard deviation (n=3).

Effect of electron donor on molybdate reduction

Of all the tested electron donors, glucose proved to be the best electron donor for supporting molybdate reduction. This was subsequently followed by descending order of sucrose, adonitol,

mannose, maltose, mannitol glycerol, salicin, myo-inositol, sorbitol and trehalose (**Fig. 5**). Previous works demonstrated that several of Mo-reducing bacteria such as *E. cloacae* strain 48 [28] and *S. marcescens* strain Dr.Y9 [17] showed sucrose as the best carbon source. Other molybdenum reducers such as *E. coli* K12 [34] and *Pseudomonas* sp. strain DRY1 [35], prefer glucose as the carbon source while *Klebsiella oxytoca* strain hkeem prefers fructose [37].

The media containing carbon sources enabled the bacteria to produce the electron donating substrates, NADH and NADPH through metabolic pathways such as glycolysis, Kreb's cycle and electron transport chain. NADH and NADPH are both responsible for playing the role of electron donating substrates for the molybdenum reducing-enzyme [32].

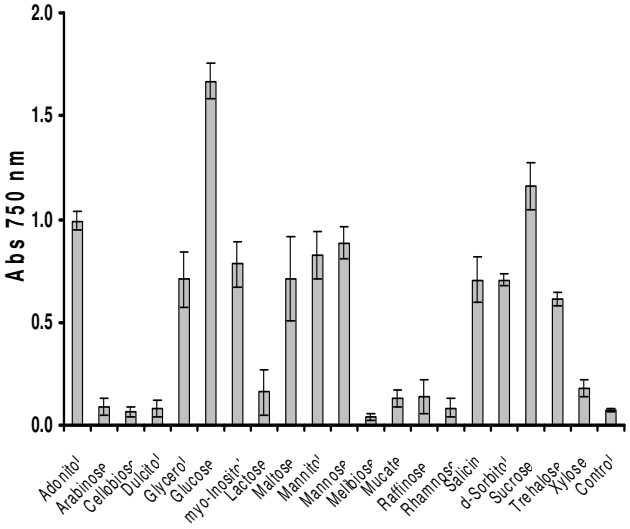

Fig. 5. Effect of different electron donor sources (1% w/v) on molybdenum reduction. Error bars represent mean ± standard deviation (n = 3).

Effect of phosphate and molybdate concentrations to molybdate reduction

Both anions (phosphate and molybdate) have been shown to inhibit Mo-blue production in bacteria, suggesting the importance of their concentrations that support molybdenum reduction [38]. The optimum concentration of phosphate occurred at 5 mM with higher concentrations were strongly inhibitory to reduction (**Fig. 6**). It was suggested that a high phosphate can inhibit the stability of phosphomolybdate complex. The complex is less stable at pH higher than neutral. Higher concentrations of phosphate produce a stronger buffering power of the phosphate buffer that is used. The phosphomolybdate complex also becomes unstable in the presence of high phosphate through a mechanism not yet known to researchers [39–41].

All of the molybdenum-reducing bacterium isolated so far requires phosphate concentration no higher than 5 mM for optimal reduction [35,36,42]. Reports on molybdenum concentration affecting molybdenum reduction demonstrated the ability of the newly isolated bacterium to reduce molybdenum as high as 60 mM with reduced Mo-blue production. The optimal reduction range was between 10 and 30 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 [32], whilst the highest molybdenum required for optimal reduction was 80 mM in *E. coli* K12 [34] and *Klebsiella oxytoca* strain hkeem [37].

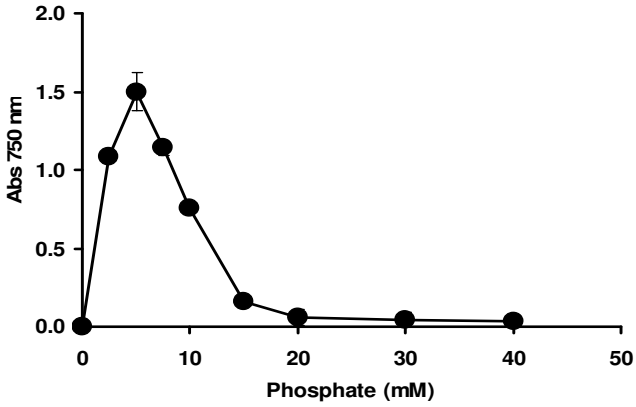

Fig. 6. The effect of phosphate concentration on molybdenum reduction by *Serratia marcescens* strain DR.Y10. Error bars represent mean ± standard deviation (n = 3).

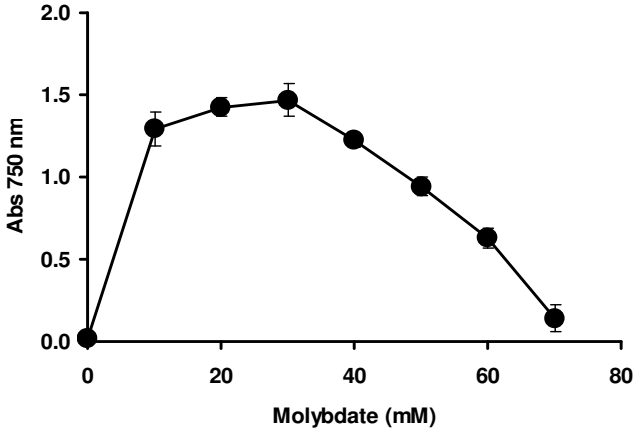

Fig. 7. The effect of molybdate concentration on molybdenum reduction by *Serratia marcescens* strain DR.Y10. Error bars represent mean ± standard deviation (n = 3).

Effect of heavy metals

Inhibition of molybdenum reduction using several metals at 1 ppm showed that Hg (ii), Ag (i), Cu (ii), and Cr (vi) inhibited molybdenum-reduction by 78.9, 69.2, 59.5 and 40.1%, respectively (**Fig. 8**). It is important to screen and isolate bacteria with as many metal resistance capability as possible since the inhibition effects by other metal ions and heavy metals demonstrate a major problem in site bioremediation. Mercury is a physiological inhibitor to molybdate reduction as previously described by Shukor *et al.,* (2002). Binding of heavy metals inactivated metal-reducing capability of the enzyme(s) responsible for the reduction.

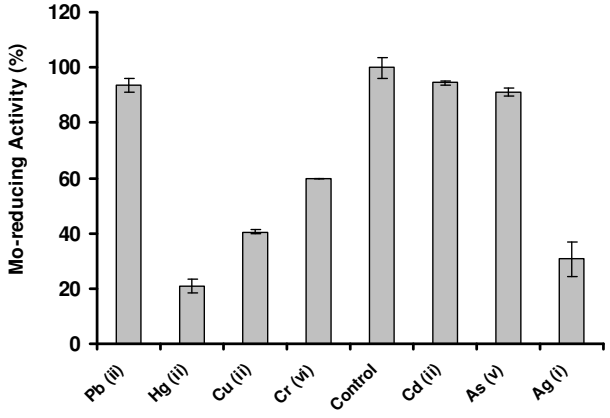

Fig. 8. The effect of metals on Mo-blue production by *Serratia marcescens* strain DR.Y10. Error bars represent mean ± standard deviation (n = 3).

Xenobiotics as electron donors for molybdenum reduction and independent growth

The ability of various xenobiotics in supporting molybdenum was carried out under static conditions. Of all the xenobiotics tested, acrylamide was shown to support molybdenum reduction at a lower efficiency than glucose while other xenobiotics tested could not (**Fig. 9**). However, acrylamide, acetamide and propionamide could support the growth of this bacterium independently of molybdenum reduction (**Fig. 10**).

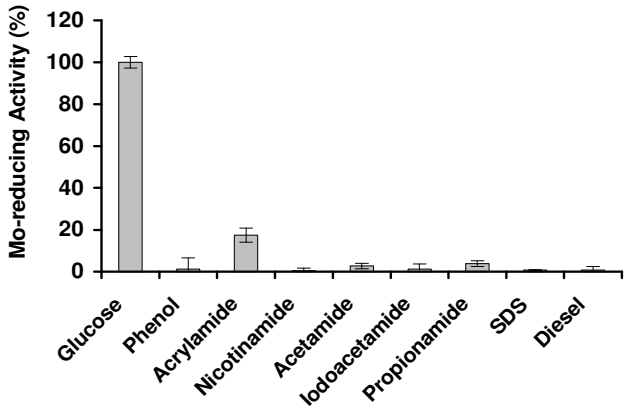

Fig. 9. Mo-blue reduction by xenobiotics at 10 mM in low phosphate media. Glucose was a positive control. Error bars represent mean ± standard deviation (n = 3).

This is the first report on carbon sources other than carbohydrates that could support Mo-reduction in the bacterium. In chromate reduction, xenobiotics such as phenol could be used as electron donors [43]. Millions of tonnes of amides such as acrylamide, acetamide and propionamide are produced annually [44]. Water-soluble thickeners that form the inert ingredient of glyphosate formulations are some of the uses of polyacrylamides synthesized from acrylamide [45]. They are also being used in wastewater treatment, papermaking, ore processing, tertiary oil recovery as well as the manufacture of permanent press fabrics. Propionamide is used as an ingredient in many different organic processes to form other useful compounds, while acetamide is used as a plasticizer and as an industrial solvent. There have been reports regarding the pollution of these amides that further leads to the isolation of several microbes, which could benefit from these amides as their carbon and nitrogen source for growth [46–48,51].

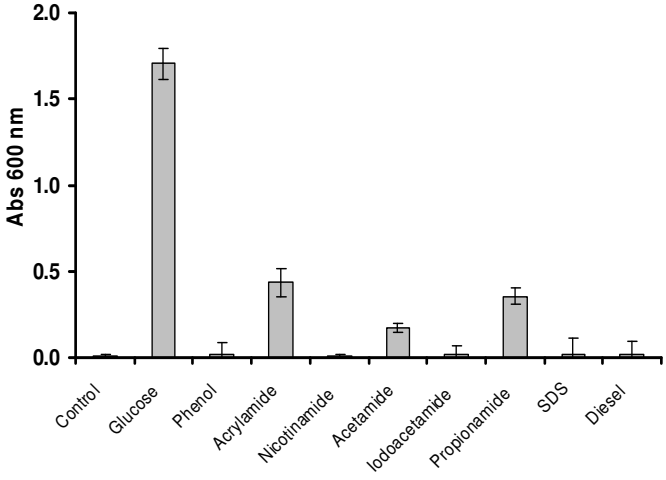

Fig. 10. The growth of *Serratia marcescens* strain DR.Y10 on xenobiotics independent of molybdenum reduction. Glucose was a positive control. Error bars represent mean ± standard deviation (n = 3).

CONCLUSION

A local isolate of the Mo-reducing bacterium with the unique ability to use acrylamide as the source of electron donor was isolated. From the results of phylogenetic analysis, the bacterium was identified as *Serratia marcescens* strain DR.Y10. The bacterium was also able to grow on amides such as acrylamide, propionamide and acetamide, but without molybdenum reduction. In Malaysia, the laws banning the importation of foreign microbes and Genetically Modified Organisms (GMOs) for bioremediation of xenobiotics are being put into effect by the government. As of such, we regard the current isolate which has the ability of detoxifying many toxicants as a promising future in bioremediation in Malaysia to be used for contaminated lands and aquatic bodies. Consequently, research has been well underway in purifying the enzyme for reducing molybdenum from this bacterium in addition to fully identifying and characterizing its xenobiotic-degrading property.

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