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Characterization of a Molybdenum-reducing *Acinetobacter baumannii* strain Serdang 1 with the Capacity to Grow on Phenol and Acrylamide

Yadzir, Z.H.M.[1], Gafar A.A[1], Yakasai, MH[2], Rahman, M.F.[1], Abdullah, M.A.[3], Shamaan, N.A.[4], Halmi, M.I.E.[5]*

[1]Department of Biochemistry, Faculty of Biotechnology and Bimolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[2]Department of Biochemistry, Faculty of Basic Medical Science, College of Health Sciences, Bayero University Kano, P.M.B 3011, Nigeria.
[3]Institute of Marine Biotechnology, University Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia.
[4]Department of Land Management, Faculty of Agriculture, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

*Corresponding author: Mohd Izuan Effendi Bin Halmi
Department of Land Management,
Faculty of Agriculture,
University Putra Malaysia,
43400 UPM Serdang,
Selangor, Malaysia.
Email: m_izuaneffendi@upm.edu.my

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ABSTRACT

Contamination of organic xenobiotic pollutants and heavy metals in a contaminated site allows the use of multiple bacterial degraders or bacteria with the ability to detoxify numerous toxicants at the same time. A previously isolated SDS- degrading bacterium, *Acinetobacter baumannii* strain Serdang 1 was shown to reduce molybdenum to molybdenum-blue. The bacterium works optimally at pH 6.5, the temperature range between 25 and 34°C with glucose serves as the best electron donor for molybdate reduction. This bacterium required additional concentration of phosphate at 5.0 mM and molybdate between 15 and 25 mM. The absorption spectrum of the molybdenum blue obtained is similar to the molybdenum blue from other earlier reported molybdate reducing bacteria, as it resembles a reduced phosphomolybdate closely. Ag(i), As(v), Pb(ii) and Cu(ii) inhibited molybdenum reduction by 57.3, 36.8, 27.7 and 10.9%, respectively, at 1 p.p.m. Acrylamide was efficiently shown to support molybdenum reduction at a lower efficiency than glucose. Phenol, acrylamide and propionamide could support the growth of this bacterium independently of molybdenum reduction. This bacterium capability to detoxify several toxicants is an important tool for bioremediation in the tropical region.

INTRODUCTION

Bacteria with the ability to detoxify more than a few toxicants including toxic heavy metals is very advantageous to remediate sites co-contaminated with several pollutants [1]. Molybdenum is an important heavy metal and forms cofactor in many enzymes including nitrate- and nitrite reductases. Molybdenum has been used as an alloying agent, lubricant, anti-corrosive component, and as anti-freeze component in the automobile engine. Its wide application in the industry has resulted in several pollutions reported worldwide including in the Tokyo Bay, Tyrol, Austria, and the Black Sea [2]. What is alarming is that recently, molybdenum has been demonstrated to inhibit spermatogenesis in several organisms like catfish and mice and can arrest embryogenesis at concentrations as low as several p.p.m. [3,4]. Classically, molybdenum affects ruminants, with cows being the most affected by disrupting the copper metabolism, and toxicity level has been a reported at several parts per million [5,6]. Aside from heavy metals, organic pollutants are major global pollutants [7,8]. An incredible number of tonnes of such chemical compounds are created every year with a good portion observed damaging the surroundings. The removal of heavy metals and organic contaminants by bioremediation is considered the most cost-effective solution in comparison with physical or chemicals approaches as this strategy may not be economical for extended terms and also primarily at low concentrations of toxicants [1].

Acrylamide is a xenobiotic toxic chemical substance which is revealed to indicate neurotoxicity in man, and demonstrated carcinogenicity and reproductive poisoning in animal models [9]. It forms the starting compound as a monomer for the polymer polyacrylamide, which is globally used in pesticides formulation, soil stabilizer and water treatment [10] and it is

from these industries that the pollution caused by acrylamide has been reported with contaminating levels as high as 1000 mg/L [11]. A known possible source of potential pollution of acrylamide in Malaysia can be contributed from the treatment of drinking water [10].

Microorganisms which could break down many xenobiotics substance are in great requirement as the flexibility of these microorganisms are required in contaminated sites in which several contaminations are present [12]. Xenobiotic degradation together with heavy metals reduction has been reported [13,14]. Previously, molybdenum-reducing bacteria that grow on sodium dodecyl sulfate (SDS) as the main carbon source have been reported although molybdenum reduction was not supported by SDS [15]. Another Mo-reducing bacterium can also use amide as an electron donor source for molybdenum reduction [16], and this activity is the most sought after regarding remediating sites contaminated with amides and heavy metals.

In this work, a previously isolated phenol degrading bacterium [17] were screened for the capacity to reduce molybdenum to molybdenum blue and to evaluate numerous xenobiotics capacity to perform as electron donors in molybdenum reduction. On top of that, the bacterium capacity to utilize these xenobiotics separately as a carbon source for growth were furthermore screened [18]. The fact that this bacterium can reduce molybdenum and grow on two toxic xenobiotics is novel.

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).

Growth and maintenance of molybdenum-reducing bacterium

Acinetobacter baumannii strain Serdang 1 was grown and maintained on a low phosphate medium (LPM) with the following compositions: 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) [19]. Blue colonies growth on the plate indicate molybdenum reduction by bacteria. Molybdenum reduction in liquid media was conducted in 100 mL of the above LPM media with the phosphate concentration increased to 100 mM in 250-mL shake flasks at pH 7.0 at room temperature for 48 h of incubation at 120 rpm orbital shaker speed. Molybdenum blue spectrum was monitored by scanning the absorption spectrum from 400 to 900 nm (Shimadzu 1201UV-Vis).

Preparation of resting cells for molybdenum reduction characterization

Characterization of the molybdenum reduction to molybdenum blue was conducted using the resting cell as before in a microtiter plate format [20]. Molybdenum blue production at the defined time was measured using the specific extinction coefficient of 11.69 $mM.^{-1}.cm^{-1}$ at 750 nm [36].

Screening of molybdenum reduction using xenobiotics as source of electron donor for molybdenum reduction and growth

Xenobiotic compounds like phenol, acrylamide, nicotinamide, acetamide, acetamide, iodoacetamide, propionamide, Sodium Dodecyl Sulfate (SDS) and diesel ability to act as electron donors in molybdenum reduction was tested using the microplate format by replacing glucose with these xenobiotics compound in low phosphate medium at the final concentration of 500 mg/L. Diesel was added at the final concentration of 0.5 g/L in 10 mL media and sonicated for 5 minutes. Two hundred µL of the media was then added to the microplate wells. To screen for the ability of these toxicants to be utilized as a carbon source of growth independent of molybdenum reduction, molybdenum was omitted from the LPM media and growth was monitored through the increase in absorption at 600 nm.

Effect of heavy metals on molybdenum reduction

Seven heavy metals; Pb (ii), As (v), Cu (ii), Hg (ii), Ag (i), Cr (vi) and Cd (ii) were studied in this work. Bacterial incubation with heavy metals was conducted in a microtiter plate at the final concentration of 1 p.p.m. and incubated for 48 h at room temperature. Molybdenum blue production was measured 750 nm as before.

Statistical analysis

All data were analyses using GraphPad InStat version 3.05 and GraphPad Prism version 3.0 available from www.graphpad.com. Student's t-test analysis or one-way analysis of variance with post hoc analysis by Tukey's test was conducted for comparing between groups. $P < 0.05$ was measured as statistically significant.

RESULTS AND DISCUSSION

A simple and rapid high throughput method was use in this work involving microplate format to obtaining more data and to speed up characterization when compare to normal shake flask approach [20,38]. Resting cells in stationary condition have been used to characterize molybdenum reduction in bacterium was commenced by [22]. Heavy metals reduction study involving resting cells had been used in selenate [39] and SDS biodegradation SDS [40].

Molybdenum absorbance spectrum

Acinetobacter baumannii strain Serdang 1 exhibited absorption spectrum of molybdenum blue at about 700 nm with a maximum peak close to infra-red region (860 nm and 870 nm) with median recorded at 865 nm (**Fig. 2**). Molybdenum blue identity was not easily determined because of it complex structure and many species [23]. Molybdenum blue is a reduced product of molybdenum complexes- isopolymolybdate and heteropolymolybdate. According to Campbell et al. [21] molybdenum blue reduction observe in *E. coli* K12 is a reduced form of phosphomolybdate without no reasonable mechanism. Isopolymolybdenum blue formation from molybdate is not likely to happen by biological-based reducing agents as a strong reducing agent was required for the conversion to happen under acidic condition. Heteropoly molybdenum blue formation by biologically-based reducing agents like ascorbic acids or enzymatic reduction is more likely as observe in the phosphate determination method by means of ascorbic acid [41]. The hypothesis is that microbial molybdate reduction in molybdate containing media must progress through phosphomolybdate intermediate. Molybdate conversion to this structure happen due pH decrease during bacterial growth. To put it simply, both chemical and biological processes are required in molybdenum reduction to molybdenum blue.

Molybdenum blue absorption spectrum from this bacterium must show a spectrum closely similar to phosphate determination method if it follows this mechanism. To be exact, maximum absorption spectrum observed must in between 860

nm and 870 nm take up at approximately 700 nm. Phosphate determination method for molybdenum blue usually display maximum absorption about 880 to 890 nm and taken up around 700 nm to 720 nm [28]. Previously we have shown that the whole Mo-blue spectra from other bacteria follow this condition [23]. Absorption spectrum result from this work clearly indicates similar spectrum pattern thus provides substantial evidence as a hypothesis. Exact phosphomolybdate species identification must be conceded out using n.m.r and e.s.r. due to the compound-complex structure. Characterization of heteropolymolybdate species through spectrophotometric analysis by examining the scanning spectroscopic profile is a less cumbersome and accepted method [42]. Maximum absorption for molybdenum blue was at b65 nm, however, measurement at 750 nm although it is 30% lower than normal, it is enough for molybdenum blue production routine monitoring as the intensity obtained was higher than cellular absorption at 600-620 nm [20]. Previously several wavelengths like 710 nm [22] and 820 nm [21] were used for molybdenum blue production monitoring.

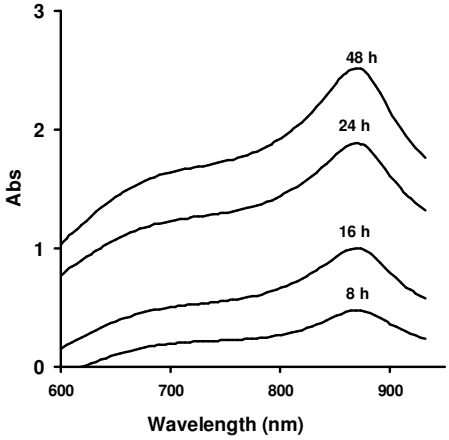

Fig. 2. Scanning absorption spectrum of Mo-blue from *Acinetobacter baumannii* strain Serdang 1 at different time intervals.

Effect of pH and temperature on molybdate reduction

Incubation of *Acinetobacter baumannii* strain Serdang 1 at different pH ranging from pH 5.5 to 8.0 with Tris-Cl and Bis-Tris buffers (20 mM) showed that ANOVA analysis for optimum pH was at 6.3 (**Fig. 3**). The effect of temperature over a wide temperature range (20 to 60°C) was observed with optimum temperatures ranging from 25-34°C with no significance different ($p>0.05$) among the measured values as analysed with ANOVA (**Fig. 4**). Molybdenum blue production from *Acinetobacter baumannii* strain Serdang 1 was inhibited strongly at temperatures lower than 34 °C.

Both pH and temperature show important roles in molybdenum reduction. As this process was mediated by an enzyme, both parameters disturbs enzyme activity, and protein folding resulted in inhibition of molybdenum reduction. In tropical country similar to Malaysia with yearly average temperature ranging from 25 to 35 ºC the optimum condition would be advantageous [24]. Therefore, *Acinetobacter baumannii* strain Serdang 1 might be a suitable candidate for molybdenum soil bioremediation locally and in other tropical countries. Common reducers show an optimum temperature ranges in between 25, and 37 ºC [19,24,25,27–31,33–35,43] as all of them were isolated from tropical soils with only one psychrotolerant reducer isolated from Antarctica exhibit optimal temperature for supporting a reduction in between 15 and 20 ºC [32]. *Acinetobacter baumannii* strain Serdang 1 optimum pH ranges for molybdenum reduction indicate bacterium property as neutrophile organism. Neutrophils are characterised by their capability to grow in between pH 5.5 and 8.0. A significant observation is that the optimal pH for molybdenum reduction in bacteria is a slightly acidic pH with optimum pHs ranging from pH 5.0 to 7.0. [21,22,24–35,43]. As previously suggested, acidic pH is important as it involves phosphomolybdate formation and stability before it is being reduced to Mo-blue. Therefore, the optimal reduction happens as there is a balance between enzyme activity and substrate stability [44].

Effect of electron donor on molybdate reduction

Glucose is the best electron donor for molybdate reduction followed by citrate (**Table 1**) among all others electron donor tested as others carbon sources cannot support molybdenum reduction. As describe previously by Shukor et al. some molybdenum reducing bacteria like S. marcescens strain Dr.Y9 [19], Serratia sp. strain Dr.Y5 [25], Enterobacter cloacae strain 48 [22], and Serratia marcescens strain DRY6 [24] indicated sucrose as the best carbon source.

Table. 1. Effect of various sources of electron donor (1% w/v) on molybdenum reduction by *Acinetobacter baumannii* strain Serdang 1. Error bars represent mean ± standard deviation (n = 3).

Carbon source	A 750 nm (mean±SD, n=3)		
Citrate	0.323	±	0.013
Fructose	0.108	±	0.067
D-Glucose	0.892	±	0.088
Glycerol	0.131	±	0.098
Glycogen	0.102	±	0.014
meso-Inositol	0.101	±	0.096
Lactose	0.111	±	0.059
Mannitol	0.122	±	0.101
D-Mannose	0.069	±	0.007
Maltose	0.089	±	0.043
Melezitose	0.121	±	0.103
Melibiose	0.091	±	0.049
Raffinose	0.103	±	0.067
Rhamnose	0.074	±	0.025
Ribose	0.115	±	0.098
Salicin	0.076	±	0.006
Sorbitol	0.105	±	0.103
Sucrose	0.088	±	0.008
Starch	0.086	±	0.100
Trehalose	0.083	±	0.097
Control	0.126	±	0.000

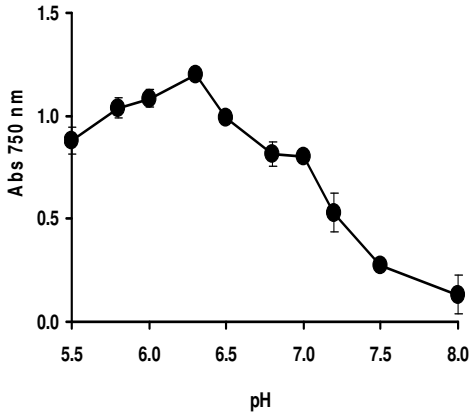

Fig. 3. Effect of pH on molybdenum reduction by *Acinetobacter baumannii* strain Serdang 1. The bacterial resting cell was incubated in a microtiter plate under optimized conditions for 72 hours. Error bars represent mean ± standard deviation (n=3).

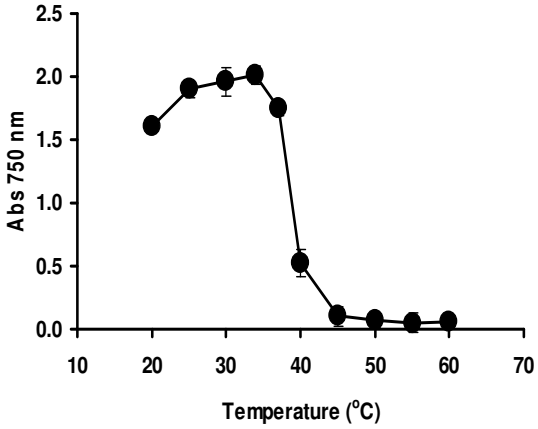

Fig. 4. Effect of temperature on molybdenum reduction by *Acinetobacter baumannii* strain Serdang 1. The bacterial resting cell was incubated in a microtiter plate under optimized conditions for 72 hours. Error bars represent mean ± standard deviation (n=3).

For instance, *Bacillus* sp. strain A.rzi [34], *Pseudomonas* sp. strain DRY1 [32], *Escherichia coli* K12 [21], *Bacillus pumilus* strain lbna [31], *Serratia* sp. strain Dr.Y5 [25], *Enterobacter* sp. strain Dr.Y13 [26] *Pseudomonas* sp. strain DRY2 [28], and *Acinetobacter calcoaceticus* strain Dr.Y12 [29] all prefer glucose as the carbon source. Carbon sources presence in the media, allow the bacteria to produce electron donating substrates like NADH and NADPH through various metabolic pathways like glycolysis, electron transport chain and Kreb's cycle. Both NADPH and NADH are in control of electron donating substrates for molybdenum reducing-enzyme [43,45].

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 displayed inhibition of molybdenum blue production in bacteria.[19,24,26–30,32,34,43]. Phosphate concentration was optimum at 5 mM with higher concentrations strongly inhibit to reduction (**Fig. 5**). High phosphate concentration inhibits phosphomolybdate stability as this complex needs acidic conditions. An increase in phosphate concentration allows strong buffering power of the phosphate buffer used. Moreover, the presence of high phosphate through unknown mechanism make phosphomolybdate complex unstable [46–48]. So far, all isolated molybdenum-reducing bacterium obliges to have not more than 5 mM phosphate concentration for optimal reduction [21,22,24–35,43].

Effect of molybdenum concentration studies on molybdenum reduction exhibited that, the newly isolated bacterium was able to reduce molybdenum as high as 60 mM but with a decrease in molybdenum blue production. The reduction was optimum at a range between 15 and 25 mM (**Fig. 6**). High concentration reduction to insoluble form allow concentrated molybdenum pollution to be reduced. The highest molybdenum concentration for optimal reduction in both *E. coli* K12 [21] and *Klebsiella oxytoca* strain hkeem [30] is 80 mM whilst 15 mM of molybdenum concentration reported in *Pseudomonas* sp strain Dr.Y2 is the lowest optimum concentration [28]. Other molybdenum- reducing bacteria like EC48 [22] and *Enterobacter* sp. strain Dr.Y13[26] both can produce optimal molybdenum blue at 50 mM molybdate concentration. More than that both *S. marcescens* strain Dr.Y6 [24] and *S. marcescens*. Dr.Y9 [19] reported to tolerate molybdate concentrations at 25 mM and 50 mM respectively. A few others reported species like *Pseudomonas* sp. strain Dr.Y2 [28], *Serratia* sp. strain Dr.Y5 [25], and *Acinetobacter calcoaceticus* [29] was reported to tolerate 30 mM, 30 mM and 20 mM of optimal molybdate concentration respectively. This show that potential bacteria strain that can tolerate high concentration of molybdenum pollution in real environment have been isolate as currently the highest molybdenum concentration reported in the environment is at 2000 ppm near to 20 mM [49].

Effect of heavy metals

Molybdenum reduction was inhibited by Ag (i), As (v), Pb (ii) and Cu (ii) at 57.3, 36.8, 27.7 and 10.9%, respectively, at 1 ppm (**Fig.** 8). In bioremediation process, a main problem is the inhibition by other metals ions and heavy metals that might affects the whole bioremediation process. Therefore, the need to screen and isolate bacteria with many metal resistance capabilities is important. In a previous work, Shukor et.al. [50], describe mercury as a physiological inhibitor to molybdate reduction while in this work, the metal appears not to inhibite reduction. Most molybdenum-reducing bacterium were inhibited by toxic heavy metals (**Table 1**). Typical heavy metals like mercury, silver, cadmium and copper naturally target sulfhydryl group of enzymes [51]. Other heavy metals like chromate is popularly known to inhibit glucose oxidase enzyme [52]. Binding of heavy metals inactivated metal-reducing capability of the enzyme(s) responsible for the reduction.

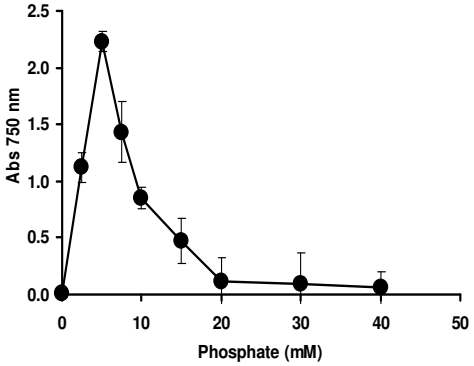

Fig. 5. The effect of phosphate concentration on molybdenum reduction by *Acinetobacter baumannii* strain Serdang 1. Bacterium resting cells were incubated in a microtiter plate at optimized conditions for 72 h. Error bars represent mean ± standard deviation (n = 3).

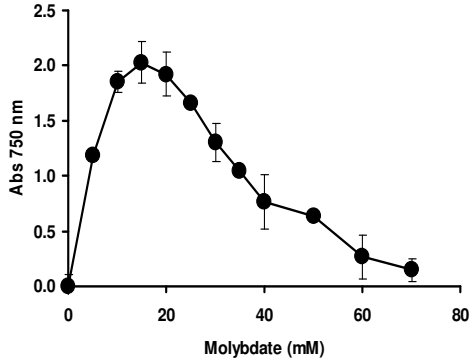

Fig. 6. The effect of molybdate concentration on molybdenum reduction by *Acinetobacter baumannii* strain Serdang 1. Bacterium resting cells were incubated in a microtiter plate at optimized conditions for 72 hours. Error bars represent mean ± standard deviation (n = 3).

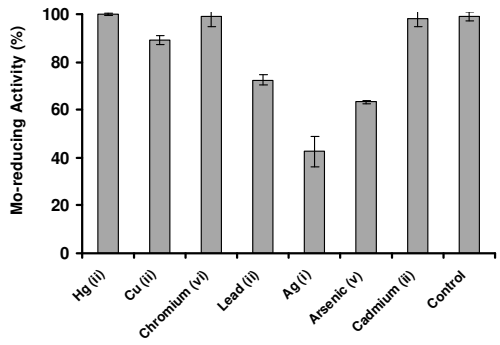

Fig. 7. The effect of metals on Mo-blue production by *Acinetobacter baumannii* strain Serdang 1. Bacterium resting cells were incubated in a microtiter plate at optimized conditions for 72 hours. Error bars represent mean ± standard deviation (n = 3).

Table 1. Inhibition of Mo-reducing bacteria by heavy metals**.**

Bacteria	Heavy Metals that inhibit reduction	Author
Bacillus pumilus strain Ibna	As^{3+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	[31]
Bacillus sp. strain A.rzi	Cd^{2+}, Cr^{6+}, Cu^{2+}, Ag^{+}, Pb^{2+}, Hg^{2+}, Co^{2+}, Zn^{2+}	[34]
Serratia sp. strain Dr.Y8	Cr, Cu, Ag, Hg	[27]
S. marcescens strain Dr.Y9	Cr^{6+}, Cu^{2+}, Ag^{+}, Hg^{2+}	[19]
Serratia sp. strain Dr.Y5	n.a.	[25]
Pseudomonas sp. strain DRY2	Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	[28]
Pseudomonas sp. strain DRY1	Cd^{2+}, Cr^{6+}, Cu^{2+}, Ag^{+}, Pb^{2+}, Hg^{2+}	[32]
Enterobacter sp. strain Dr.Y13	Cr^{6+}, Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}	[26]
Acinetobacter calcoaceticus strain Dr.Y12	Cd^{2+}, Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	[29]
Serratia marcescens strain DRY6	Cr^{6+}, Cu^{2+}, Hg^{2+}*	[24]
Enterobacter cloacae strain 48	Cr^{6+}, Cu^{2+}	[22]
Escherichia coli K12	Cr^{6+}	[21]
Klebsiella oxytoca strain hkeem	Cu^{2+}, Ag^{+}, Hg^{2+}	[30]

Xenobiotics as electron donors for molybdenum reduction and independent growth

Various xenobiotics ability to support molybdenum reduction was explored. Out of all the xenobiotics tested, acrylamide displayed the ability to support molybdenum reduction at a lower efficiency than glucose while other xenobiotics tested cannot (**Fig. 8**). However, phenol (as expected), acrylamide and propionamide could support the growth of this bacterium independently of molybdenum reduction (**Fig. 9**). This is the first report on carbon sources other than carbohydrates that could support Mo-reduction in the bacterium. Other xenobiotics like phenol can be used as electron donors in chromate reduction [53]. Amides such as acrylamide, acetamide and propionamide are produced in the order of millions of tons per year [54]. Acrylamide is mostly used to produce the polyacrylamides polymer that has several uses as water-soluble thickeners and as an inert ingredient of various glyphosate formulations [55]. Other uses include in the papermaking process, as coagulants in wastewater treatment and as permanent fabrics press in cloth manufacturing process. Acetamide is used as a plasticizer and as an industrial solvent while propionamide is used as an ingredient in many different organic processes to form other useful compounds. The pollution of these amides have been reported, and several microbes have been isolated that could use these amides growth sources of nitrogen and carbon.[10,10,54,56,56–58,58–66].

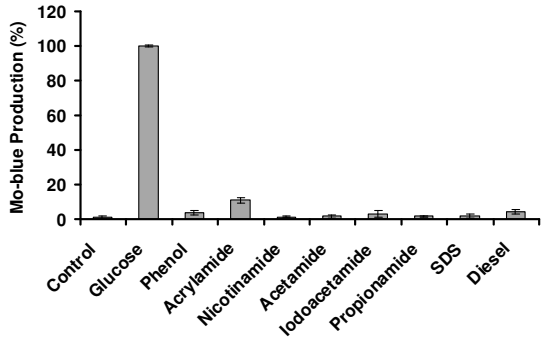

Fig. 8. Mo-blue reduction by xenobiotics at 10 mM in low phosphate media. Glucose was a positive control. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 72 hours. Error bars represent mean ± standard deviation (n = 3).

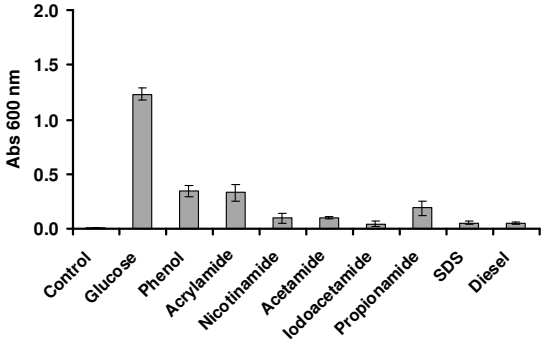

Fig. 9. The growth of *Acinetobacter baumannii* strain Serdang 1 on xenobiotics independent of molybdenum reduction. Glucose was a positive control. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 72 hours. Error bars represent mean ± standard deviation (n = 3).

CONCLUSION

A phenol-degrading bacterium has shown to be able to reduce molybdenum to molybdenum, blue. The bacterium works optimally to reduces molybdate to molybdenum blue at pH 6.3 and temperatures ranges between 25° C and 34 ° C. Glucose act as the best electron donor for molybdate reduction followed by citrate. Additional phosphate concentration at 5.0 mM and molybdate concentration at between 15 and 25 mM are required. Molybdenum-blue produces similar absorption spectrum compared to the previous Mo-reducing bacterium and resembles a reduced phosphomolybdate closely. Molybdenum reduction was inhibited by Ag (i), As (v), Pb (ii) and Cu (ii) at 57.3, 36.8, 27.7 and 10.9%, respectively. Acrylamide was shown to support molybdenum reduction at a lower efficiency than glucose while other xenobiotics tested could not. Phenol, acrylamide and propionamide can support this bacterium growth independent of molybdenum reduction. This bacterium has the ability to detoxify numerous toxicants, and this is a novel property, and this makes the bacterium an important tool for bioremediation in the tropical region. At present, efforts are

ongoing to purify the molybdenum-reducing enzyme from this bacterium and to fully characterize its xenobiotic-degrading property.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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