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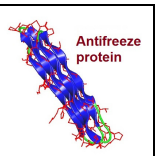

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Isolation of Molybdenum-reducing Bacterium; *Serratia* sp. strain MIE2 from Agriculture Soil and its Potential Use in Soil Bioremediation

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ABSTRACT

Molybdenum is reported to be very toxic to ruminants and shows evidence of spermatogenesis toxicity in animals and insects. Hence, its removal is important. In this study, we report on the first isolation of molybdenum-reducing bacterium from agricultural soil. The bacterium reduces hexavalent molybdenum (sodium molybdate) to molybdenum blue (Mo-blue); a colloidal product, which can be trapped and removed from solution. Phylogenetic analysis resulted in a tentative identification of the bacterium as *Serratia* sp. strain MIE2. The optimum conditions for Mo-blue production using the normal one-variable-at-a-time (OVAT) approach were 10 mM of sodium molybdate, pH 6.0, a temperature of 35°C, ammonium sulphate at 10 g/L as the nitrogen source and sucrose concentrations of between 30-50 g/L as the carbon source and electron donor for molybdate. Studies on the effects of pesticides and solvents on Mo-blue production showed that Mo-blue production from whole cells was relatively more affected by these xenobiotics compared to the crude enzyme. Nevertheless, the strain was resistant to most of the xenobiotics tested. Based on the strain MIE characteristics, the bacterium will be a suitable candidate for the remediation of aquatic bodies and agricultural soils contaminated with molybdenum.

INTRODUCTION

Molybdenum is a crucial microelement for microorganisms, plants and animals because it is a constituent of numerous enzymes [1]. It has five possible oxidation states with molybdate (VI) being the common soluble form in the environment. In dilute solutions with pH near to neutral, the main form of the soluble molybdenum is the molybdate anion, which is reported as the only bioavailable form for plants. Under acidic conditions, molybdate ion is easily polymerized into polyions, which include $Mo_8O_{26}^{4-}$ $Mo_7O_{24}^{6-}$, and $Mo_{12}O_{37}^{2-}$. Mild reducing agents such as stannous ions, ascorbate and dithionate reduces these ions forming "isopolymolybdenum blue". Another unique property of these polysions is that they can form further complex structure through coordinating with the heteroatoms arsenate, phosphate, sulphate, tungstate, and silicate resulting in the heteropolymolybdates arsenomolybdate, phosphomolybdate, sulphomolybdate, tungstomolybdate, and silicomolybdate respectively. These heteropolymolybdates can also be reduced by the same reducing agents forming molybdenum blue, which can be distinguished from the isopolymolybdates through their scanning absorption spectra [2].

Molybdenum-containing wastes could be released to the ecosystem from the utilization of molybdenum as catalysts, alloys, lubricants, and also through mining activities. Molybdenum can be found in soil at between 0.2 and 6 mg/kg, while soils and aquatic bodies polluted with molybdenum can contain up to 6,600 mg/kg [3-4]. Ruminants grazing on molybdenum-polluted soil have been reported to suffer from hypocuprosis leading to scouring and death [5]. Molybdenum has also been reported to inhibit spermatogenesis in drosophila [6] and fish [7].

In order to remove toxic metals including molybdenum from the environment, bioremediation is one of the safest approaches [8-9]. Amongst the first reported molybdenum bioremediation was performed in Tyrol, Austria upon agricultural soil exposed for years to molybdenum [5]. The application of phytoremediation and a mixture of microbes isolated from the soil itself managed to immobilize the molybdate into non-soluble form, ultimately reducing its toxicity. To date, there are numerous reports on the reduction of molybdate by microbes [10-20]. All of these bacterial reductions of molybdate concerns the reduction of molybdenum in the form of molybdate (Mo^{6+}) into the lower oxidation state molybdenum blue with a complex oxidation state of between 5+ and 6+ [21]. This reduction is a part of detoxification strategy [11].

The reduction process in molybdenum-reducing bacteria in general has been proven to be an enzymatic reaction and not chemically mediated [13]. Molybdenum-reducing bacteria from agricultural soil have been reported [22], with a second molybdenum-reducing bacterium exhibiting glyphosate-degrading capability [20].
As molybdenum is very toxic to ruminants, with deaths upon consuming feeds containing as low as 20 p.p.m molybdenum [5], there is a need for more molybdenum reducer isolating from agriculture soil for bioremediation process. In addition, toxicity of the bacterium and its molybdenum-reducing capacity to pesticides needs to be assessed as agricultural soils are usually contaminated with these compounds.

In this study we report on the optimization of Mo-blue production from bacterium using OVAT approach. We also report on the first study of the effect of solvents and pesticides on Mo-blue production; a study that is important when considering the use of the Mo-reducing bacterium for remediation of molybdenum in agricultural soils.

MATERIALS AND METHODS

Statistical analysis

Statistical analyses were carried out using GraphPad Prism 5.0 (available from www.graphpad.com).

Isolation of molybdenum-reducing bacteria

Soil samples were collected from agriculture areas in Universiti Putra Malaysia in 2012, and were placed in sterile containers and immediately transferred to the laboratory. One gram of soil sample was suspended in low phosphate media (LPM) containing 10 mM sodium molybdate and incubated for 24 h at 30°C on an orbital shaker (120 rpm, Yihder, Taiwan). The ingredient of the media was as follows: $(NH_4)2{\bullet}SO_4$ (0.3%), glucose (1%), NaCl (0.5%), $MgSO_4{\bullet}7H_2O$ (0.05%), $Na_2MoO_4{\bullet}2H_2O$ (0.24 %), yeast extract (0.5%),and Na_2HPO_4 (0.04%) [23]. The soil suspension was pipetted (50 µl aliquot) and spread onto a solid on low phosphate media or LPM (pH 7.5). After two days of incubation at room temperature, the formation of blue colonies shows the presence of molybdenum-reducing bacteria.

The colony exhibiting the strongest blue intensity was then re-streaked in a fresh LPM agar. To study the characteristics of molybdate reduction by the isolate, the colony was transferred into 100 mL of high phosphate medium (HPM) with the phosphate concentration in the latter was increased to 100 mM (Na_2HPO_4, 1.42%) to prevent Mo-blue formation that aggregates with bacterial cells [11]. The culture was incubated for 24 h at 30°C on an orbital shaker (120 rpm, Yihder, Taiwan). Then, 2% (v/v) of this bacterial aliquot (optical density at 600 nm of between 0.9 and 1.0) was added into 100 mL of freshly prepared liquid LPM. The mixture was incubated for 24 h at 30°C to allow for molybdate reduction. Measurement of molybdenum blue was carried out at 865 nm using 16.3 $mM.^{-1}.cm^{-1}$ as the specific extinction coefficient to quantify Mo-blue.

To measure Mo-blue production, one milliliter of the molybdenum-blue formed from the liquid LPM culture was pipetted out and centrifuged (Eppendorf™) at room temperature at 10,000×g for 20 min. Absorbance value of molybdenum-blue above 1.0 was directly diluted using blank media. A second preparation of the supernatant was scanned with low phosphate media as the baseline correction from 400 to 900 nm (UV-spectrophotometer, Shimadzu 1201) [12].

The dialysis tubing method to distinguish between abiotic and biotic molybdenum reduction

The dialysis tubing method of Shukor et al. [12], which is a modification of an earlier method [24] was utilized to discriminate whether the reduction of molybdenum is an abiotic or biotic process. The bacterium was grown in 500 mL of HPM at 30°C with shaking on an orbital shaker at 120 rpm for 24 h. Cells were harvested by centrifugation at 10,000xg for 20 min. The pellet was resuspended in 30 mL of LPM (pH 7.0) at 30°C containing (w/v) yeast extract (0.05%), $MgSO_4{\bullet}7H_2O$ (0.05%), $(NH_4)2SO_4$ (0.3%), NaCl (0.5%) and $Na_2HPO_4{\bullet}2H_2O$ (0.05%). Sodium molybdate was omitted. About 20 mL of this suspension was placed into a previously boiled (10 min) dialysis tubing (12 kDa molecular weight cut-off). The dialysis tubing was then immersed in sterile 100 mL of LPM (pH 7.0) for 20 h at 30°C. Aliquots (1 mL) of the internal and external medium were periodically taken and centrifuged at 10,000 x g for 20 min. The supernatant containing Mo-blue was then read at 865 nm [15]. Experiments were carried out in triplicates.

Identification of molybdenum-reducing bacterium

A partial identification of the bacterium was carried out using 16s rRNA gene molecular phylogenetics according to the evolutionary distance matrices (neighbour-joining/UPGMA) [15]. PHYLIP, version 3.573 was utilized to construct the phylogenetic tree with the outgroup being *Bacillus thuringiensis* (accession number AY741718).

Optimization using OVAT

The effect of various parameters such as molybdate and phosphate concentrations, temperature, pH, electron donor sources and nitrogen sources on molybdate reduction by *Serratia* sp. MIE2 was studied using the LPM. Briefly, two milliliters of bacteria cells from a 24-hour bacterial culture grown in HPM at 30°C with an optimal density at 600 nm of between 0.9 and 1.0 were pipetted and transferred into 100 mL of LPM. Aliquots (1 mL) of the internal and external medium were periodically taken and centrifuged at 10,000 x g for 20 min. The supernatant containing Mo-blue was then read at 865 nm as before.

Preparation of molybdenum-reducing partially purified enzyme

Serratia sp. MIE2 was grown in 2 L of HPM for 48 h at 30°C [15]. Unless stated otherwise, experiments were carried out at 4°C. Bacterial cells from the culture media were centrifuged at 10,000 xg for 20 min. The pelleted cells were briefly rinsed with distilled water. With the aid of a glass rod, the pellets were re-suspended in 10 ml of 50 mM Tris-HCl (pH 7.0) with 2 mM of DTT and 1 mM of phenylmethane-sulphonyfluoride (PMSF) as a protease inhibitor [25].

Cells were sonicated on a Biosonik 111™ sonicator for 1 min with 3 min cooling. The total sonication time was two h and cells were ruptured on an ice bath. The sonicated fraction was first centrifuged at 10,000×g for 20 min and the supernatant were further ultra-centrifuged at 105,000×g for 60 min. The supernatant containing the crude enzyme was further fractionated using a 40 to 50% ammonium sulphate fraction. This fraction was further dialysed for ten h in 5 L of 10 mM Tris-HCl pH 7.5 buffer containing 0.1 mM dithiothreitol with stirring to remove ammonium sulphate prior to enzyme assay [25].

The effect of pesticides and solvents on molybdenum-blue production

The effect of pesticides and solvents on molybdenum-blue production from *Serratia* sp. strain MIE2 was studied using bacterial cells and crude molybdenum-reducing enzyme. In the first experiment, a 50 mL LPM containing 10 mM molybdate was supplied with 1 p.p.m. of the pesticides carbofuran, diazinon, methomyl, propoxur, malathion, parathion, trichlorfon, bendiocarb, carbaryl, dimethoate, chlorpyrifos, atrazine and simazine, and 10% (v/v) of the solvents hexane, ethyl acetate, n-hexadecane, acetonitrile, methanol, propan-1-ol, dimethyl sulfoxide, butanol and chloroform. The media was then mixed with 2% (v/v) of bacterial culture grown in HPM previously, and incubated on a rotary shaker (120 rpm) at 30ºC for 24 h. After the incubation period, a 1 mL aliquot of the medium was centrifuged at 10,000×g for 20 min. The supernatant containing Mo-blue was then read at 865 nm.

The effect of pesticides and solvents on molybdenum-blue production from crude molybdenum-reducing enzyme was studies by incubating pesticides and solvents (50 µL) with 100 µL of enzyme fraction to the final concentrations of 1 p.p.m. and 10%, respectively, and incubated for 1 h at 4ºC. Pesticides and solvents were replaced with distilled water in the control experiment. The assay for the molybdenum-reducing enzyme was carried out by mixing a 1 mL assay mixture containing sodium phosphate buffers (50 mM) with 100 µL of 50 mM phosphomolybdate, 100 µL of 400 mM NADH and 150 µL of the previously pesticides or solvents-incubated crude molybdenum-reducing enzyme fraction. The mixture was incubated at room temperature for 20 min and production of Mo-blue was determined at 865 nm.

RESULTS AND DISCUSSION

Identification of molybdenum-reducing bacterium

Strain MIE2 was a motile, Gram-negative, short rod-shaped and a facultative anaerobe (**Fig. 1**). The 16S rDNA sequence of strain MIE2 was deposited in the GeneBank under the accession no. **KF647218**. The bacterium was also deposited at the university's in the Institute of Bioscience culture collection center, Universiti Putra Malaysia under the accession ***Serratia* sp. UPMC 926**. Strain MIE2 belongs to the genus *Serratia* according to the blast results from NCBI. Phylogenetic analysis (**Fig. 2**) showed a high bootstrap value (100%) to *Serratia* sp. ES1 and *Serratia* sp. EP28 indicating that the phylogenetic relationship to the genus level was strong but not to the species level. Hence, as of now assignment to the species level cannot be done. More work is needed to identify this bacterium to the species level. Thus, this bacterium is identified tentatively as *Serratia* sp. strain MIE2.

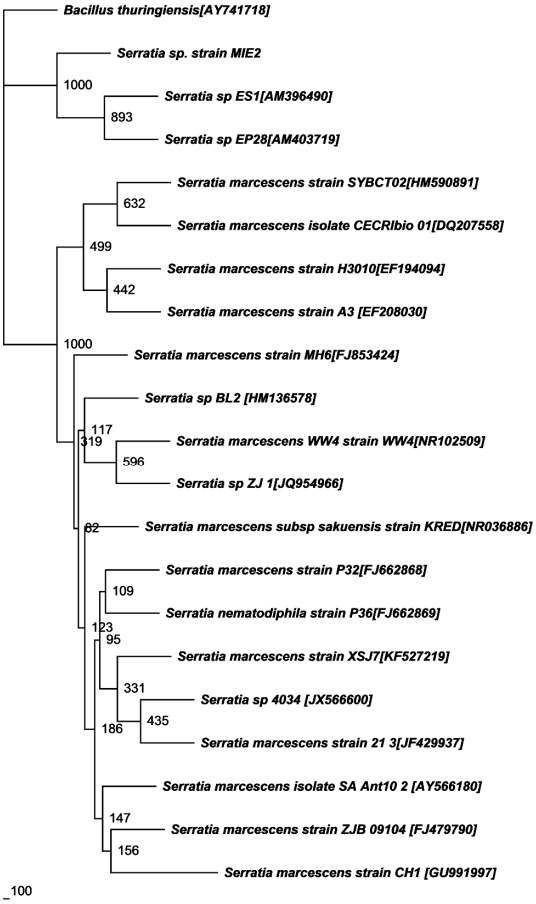

Fig. 2. Phylogram (neighbour-joining method) showing the genetic relationship between Serratia sp. strain MIE2 and other related references microorganisms based on the 16S rRNA gene sequence analysis from the GenBank database. Bacillus thuringiensis is the outgroup. Species names were followed by the accession numbers of 16S rRNA. The internal labels at the branching points refer to bootstrap value. Scale bar represents 100 nucleotides substitution.

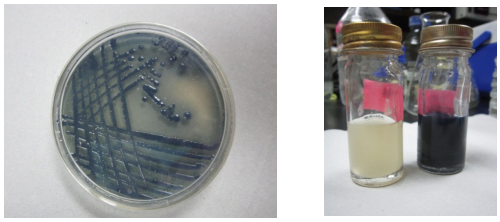

Fig. 1. a) Mo-reducing bacterium on low phosphate agar. (b) Mo-reducing bacterium in nutrient broth and low phosphate media.

Phylogenetic analysis demonstrates this bacterium belongs to the genus *Serratia* sp. Morphological and biochemical studies and molecular analysis of the 16S rRNA gene partially identifies the bacterium as *Serratia* sp. MIE2. The larger bootstrap value acquired that linked this bacterium to a few species of the *Serratia* genus indicated that further identification methods are necessary to identify this bacterium to the species level. Further identification of the bacterium can be achieved with the addition of several more polyphasic identification methods to the species level, and include methods such as the DNA-DNA hybridization, determination of genomic DNA G+C content, and fatty acid profile analysis [26].

Another molybdenum-reducing bacterium has been isolated from agriculture soil. The result is important as agricultural soil pose a great threat to ruminants if molybdenum at toxic level is present. Microbes isolated from specific areas for example, from agriculture soil might not be effective to treat pollutants in other location. In many cases, microbes isolated from specific polluted soils, grown to a large scale and reintroduced back to the polluted soils (autochthonous microbes) are better remediators than commercially available bacteria or the use of other bacterial degraders from different geographical areas (allochthonous microbes). This is the reason for the ever-increasing reporting of the isolation of novel strains of remediating microorganisms [8, 27- 32]. To date, the majority of the Mo-reducing bacterium reported in the last fifteen years came from our works.

Molybdenum blue absorbance spectrum

The molybdenum-blue produced exhibited a unique maximum peak near the infra-red region of between 860 and 870 nm and a shoulder at approximately 700 nm (**Fig. 3**).

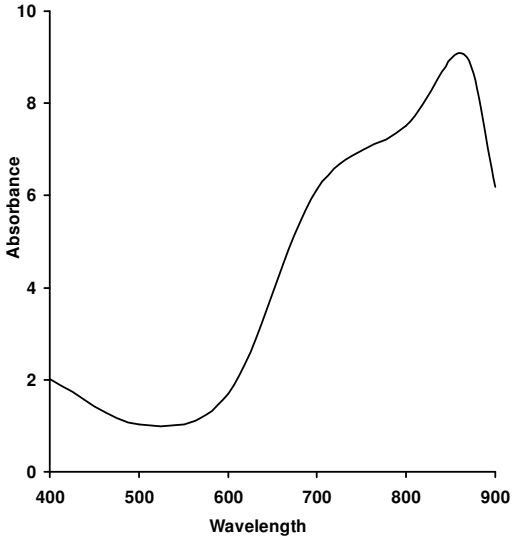

Fig. 3. Scanning spectrum of Mo-blue from *Serratia* sp. strain MIE2 after 24 h.

Characterization of the molybdate reduction reaction using the dialysis tubing method

The result showed that 98.3±1.2 % of molybdenum-blue formed was found in the dialysis tube, while only 1.2±1.1 % was found outside of the tube. The dialysis tubing is the standard technique first developed by Hem [24] to distinguish between abiotic and biotic (chemical) and enzymatic reduction of metal ions by bacteria, and has been modified to accommodate similar studies in the molybdenum reduction in bacteria [33]. Chromate reduction studies in bacteria has been plagued by the same problem with biotically-produced chemicals such as sugars and NADH have been reported to be able to reduce hexavalent chromate to lower oxidation states chromium [34]. The experiment suggests that molybdenum reduction is exclusively enzymatic in origin as the 5% of molybdenum-blue found on the outside of the tubing has been attributed to the slow leakage of the molybdenum-blue formed [13]. Similarly, in all of the previous reports using this technique, more than 90% of the molybdenum-blue formed is found inside the dialysis tubing [12- 19, 33].

Optimization of molybdenum reduction

Effect of temperature and initial pH on molybdate reduction

The bacterium was incubated at initial pHs ranging from 5.5 to 8.0. The result as presented in **Fig. 4** showed that the optimum initial pH was 6.0. The effect of temperature (**Fig. 5**) showed an optimum temperature from 27°C to 35°C with no significant difference ($p>0.05$) among the values measured. Temperatures higher than 35°C were inhibitory to molybdenum-blue production from strain MIE2. Since molybdenum reduction is enzyme mediated, both temperature and pH are anticipated to affect molybdenum reduction. The optimum temperature for molybdenum reduction by strain MIE occurs in a broad range from 27 to 35°C, and is suitable to be utilized for bioremediation of molybdenum in Malaysia and other tropical countries [15]. Strain MIE2 reduces molybdenum optimally at pH 6, indicating that this bacterium is a neutrophilic bacterium.

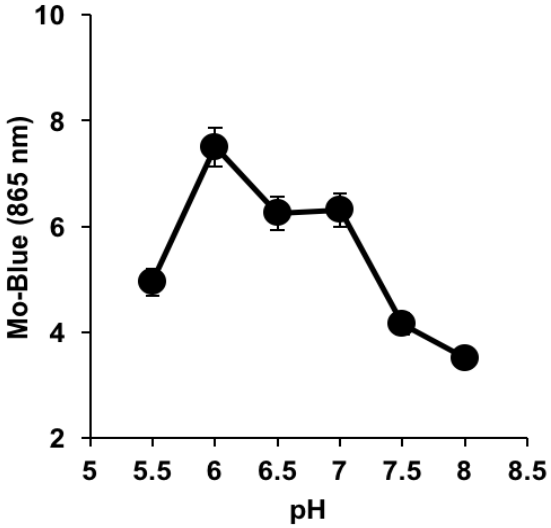

Fig. 4. Effects of initial pH on molybdenum reduction by strain MIE2. Error bars represent mean ± standard deviation (n=3).

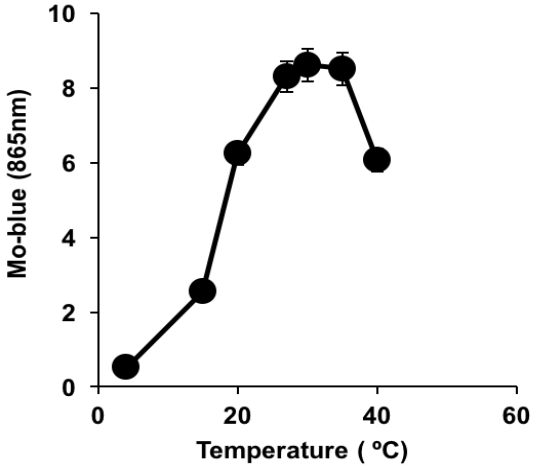

Fig. 5. Effects of temperature on molybdenum reduction by strain MIE2. Error bars represent mean ± standard deviation (n=3).

Effects of electron donor and nitrogen sources on molybdate reduction.

Among the electron donor and nitrogen sources tested, sucrose (2% w/v) was the best electron donor and ammonium sulphate (1% w/v) was the best nitrogen source for supporting molybdate

reduction (data not shown). The amount of molybdenum-blue produced was highest when strain MIE2 was grown in the medium containing sucrose followed by glucose, fructose, maltose and galactose. Starch, arabinose and raffinose did not support molybdenum-blue production. Ammonium sulphate gave significantly higher molybdenum-blue production ($p< 0.05$) followed by tryptone, ammonium chloride, glycine, L-alanine, urea, caffeine and ammonium acetate. Glutamic acid and L- glutamine did not support molybdenum-blue production (data not shown). **Fig. 6** shows that the optimum concentration of sucrose was between 30-50 g/L with no significant difference among the values ($p>0.05$) obtained.

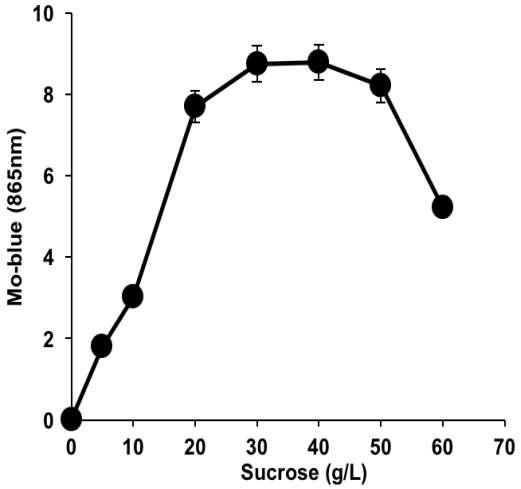

Fig. 6. Effects of different electron donor sources. Strain MIE2 was grown in low phosphate media containing 10 mM molybdate and various electron donors. Error bars represent mean ± standard deviation (n = 3)

Molybdenum reduction in bacteria to Mo-blue has been found to be best supported by easily assimilable sugars, which include glucose and sucrose. The Mo-reducing bacteria *Enterobacter cloacae* strain 48 [11], *S. marcescens* strain Dr.Y9 [14], *Serratia* sp. strain Dr.Y5 [35] and *Serratia marcescens* strain DRY6 [12] utilise sucrose as the best carbon source for molybdenum reduction. Other molybdenum reducers such as *Escherichia coli* K12 [10], *Enterobacter* sp. strain Dr.Y13 [33], *Pseudomonas* sp. strain DRY1 [17], *Acinetobacter calcoaceticus* strain Dr.Y12 [15], *Bacillus* sp. strain A.rzi [36], *Bacillus pumilus* strain lbna [16], *Klebsiella oxytoca* strain Aft-7 [37], *Pseudomonas aeruginosa* strain Amr-11 [19] and *Klebsiella oxytoca* strain Saw-5 [20] prefer glucose as the carbon source. An exception is *Klebsiella oxytoca* strain hkeem that prefers fructose as the most optimum carbon source supporting molybdate reduction although both glucose and sucrose are also utilized [23]. All of these easily assimilable carbon sources are the best carbon sources possibly because they produce the reducing equivalents NADH and NADPH, which are known substrates for the Mo-reducing enzyme. The reducing equivalents are efficiently produced through generic metabolic pathways such as Glycolysis Kreb's cycle and the electron transport system [25]. A nitrogen source is needed for the production of amino acids, enzymes and nucleic acids. Screening of nitrogen sources showed that, strain MIE2 used ammonium sulphate as the best nitrogen source. This result was in agreement with previous studies on molybdenum reduction bacteria which shows that all of the molybdenum-reducing bacteria prefer ammonium sulphate as the best nitrogen source [10-15].

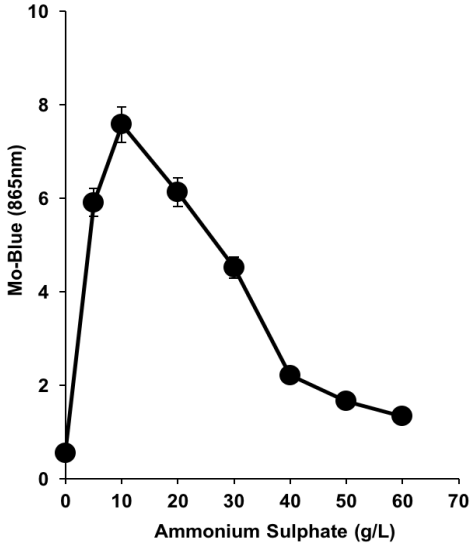

Fig. 7. Effects of different nitrogen sources. Strain MIE2 was grown in low phosphate media containing 10 mM molybdate with sucrose as electron donor. Error bars represent mean ± standard deviation (n = 3).

Effects of phosphate and molybdate concentrations on molybdate reduction

The optimum concentration of phosphate was 2 mM (**Fig. 8**). Concentration over 2 mM was inhibitory to molybdenum-blue production. The optimum concentration of molybdate occurred at 10 mM (**Fig. 9**). High phosphate and molybdate concentrations have been reported to be inhibitory to molybdenum reduction. Strain MIE2 required 2 mM phosphate for optimal reduction while 5 mM of phosphate is reported for other molybdenum-reducing bacteria [15-20]. Strain MIE2 was able to reduce molybdenum (in the form of sodium molybdate) as high as 20 mM. Reduction into an insoluble form allows the bacterium to remediate sites with high concentrations of molybdenum. To date, the highest concentration of sodium molybdate reported to be reduced by Mo-reducing bacteria is 80 mM [10, 23].

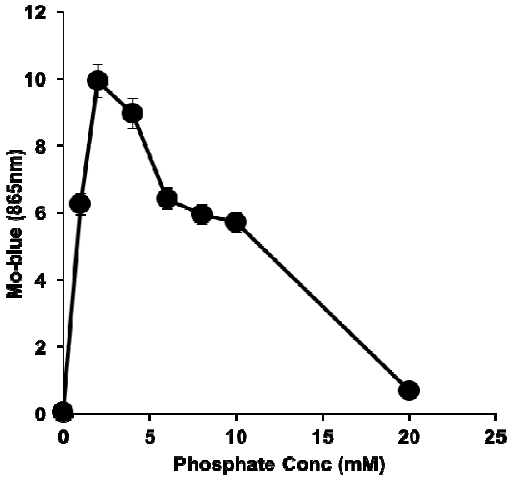

Fig. 8. The effects of phosphate concentration on molybdenum reduction by strain MIE2. Error bars represent mean ± standard deviation (n = 3).

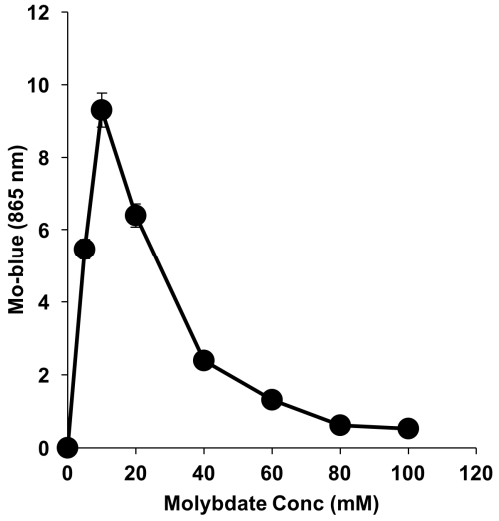

Fig. 9. The effects of molybdate on molybdenum reduction by strain MIE2. Error bars represent mean ± standard deviation (n = 3).

Effect of pesticides and solvents on molybdenum reduction

Fig. 10 shows the effect of selected pesticides to the activities of bacterial cells and molybdenum-reducing enzymes. As compared to the control, bacterial cells were inhibited by carbofuran, diazinon, methomyl, malathion, trichlorofon, bendiocarb, cabaryl with inhibition (%) less than 50% at 1 ppm. However, propoxur, parathion, dimethoate, chlorpyrifos, atrazine and simazine showed no inhibition as compared to the control. For the molybdenum-reducing enzyme, most of the pesticides gave less than 30% inhibition with diazinon, bendiocarb, cabaryl, chlorphrifos, atrazine and simazine showing no inhibition as compared to the control. Only hexane and butanol produced more than 20% inhibition to molybdenum-blue production by bacterial cells (**Fig. 11**). The other tested solvents showed less than 20% inhibition as compared to the control. Otherwise, molybdenum-reducing enzyme showed inhibition of less than 20% indicating that the enzyme was not affected much by solvents.

Pesticides are among the most persistent organic contaminants, which are of concern because of their occurrence in agricultural environment. Pesticides that interrupt the activities of soil microorganisms is expected to change the nutritional quality of soils and would consequently, have serious ecological implications and will affect bioremediation processes [38]. Therefore, the effects of pesticides to bacterial cells and molybdenum-reducing enzyme were studied to evaluate the efficiency of strain MIE2 to reduce molybdate in the presence of these inhibitors. Based on the results, the molybdenum-reducing enzyme showed a lesser inhibition compared to live bacterial cells, which could be due to the pesticides inhibiting cellular metabolic processes and affecting the metabolic process of strain MIE2 that could subsequently repress molybdenum-blue production. The effects of solvents were evaluated in study due to its application in pesticides formulation as a solvating and dispersing agent [39]. Solvents did not have much an effect on the live bacterial cells and also to the molybdenum-reducing enzyme, indicating that strain MIE2 and the enzyme are solvent tolerant, and are able to function in the presence of various tested solvents.

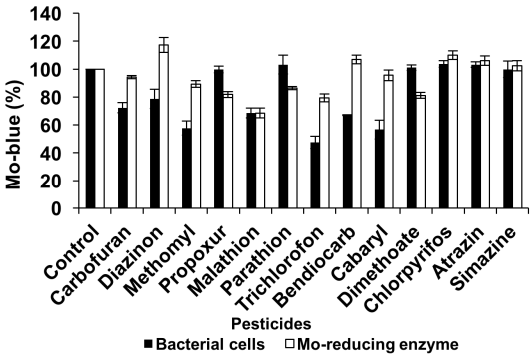

Fig. 10. The effects of pesticides to the *Serratia* sp. MIE2 bacterial cells and Mo-reducing enzyme. The error bars represent mean ± standard deviation for three replicates.

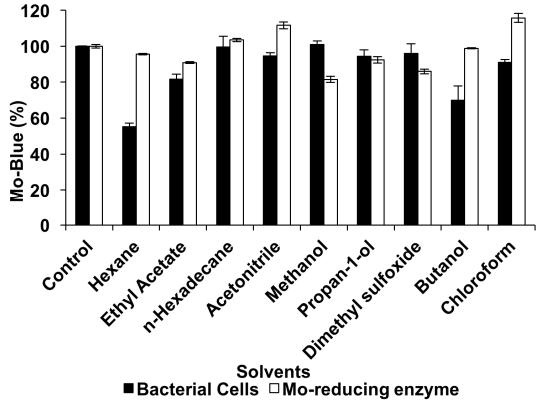

Fig. 11. The effects of solvents to the *Serratia* sp. MIE2 bacterial cells and Mo-reducing enzyme. The error bars represent mean ± standard deviation for three replicates.

CONCLUSION

In conclusion, we reported on the isolation of the first molybdenum-reducing bacterium isolated from agricultural soil. The reduction of molybdenum to molybdenum-blue is predominantly enzymatic as evident from the dialysis tubing experiment. Characterization of the reduction process was carried out through a normal approach and the highest molybdenum-blue production was obtained after the analysis. The tested pesticides and solvents show slight inhibition to production of molybdenum blue from bacterial cells. On the other hand, both types of inhibitors did not have much an effect on the molybdenum-reducing enzyme. We are currently focusing on optimization of this bacterium using response surface methodology (RSM) and purification of its molybdenum-reducing enzyme to homogeneity is ongoing

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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