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Mo (VI) Reduction to Molybdenum Blue by *Serratia marcescens* Strain Dr. Y9

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Abstract

In this work we report on the isolation of a local molybdenum-reducing bacterium. The bacterium reduced molybdate or Mo(6+) to molybdenum blue (oxidation states between 5+ to 6+). Electron donors that supported cellular growth were sucrose, maltose, mannitol, fructose, glucose and starch (in decreasing order) with sucrose supporting formation of the highest amount of molybdenum blue at 10 g/l after 24 hours of static incubation. The optimum molybdate and phosphate concentrations that supported molybdate reduction were 20 and 5 mM, respectively. Molybdate reduction was optimal at 37°C. The molybdenum blue produced from cellular reduction exhibited a unique absorption spectrum with a maximum peak at 865 nm and a shoulder at 700 nm. The isolate was tentatively identified as *S. marcescens* strain Dr.Y9 based on carbon utilization profiles using Biolog GN plates and partial 16S rDNA molecular phylogeny. No inhibition of molybdenum-reducing activity was seen using electron transport system (ETS) inhibitors such as antimycin A, [1]HQNO (Hydroxyquinoline-N-Oxide), sodium azide and cyanide suggesting that the ETS of this bacterium is not the site of molybdate reduction.

Key words: *Serratia marcescens*, molybdate-reduction, molybdenum blue

Introduction

Extensive use of metals in various industrial applications has caused substantial environmental pollution (Groudev *et al*., 2005). Some organisms are susceptible to heavy metals (Ali, 2007) while others are resistant (Lloyd, 2003; Noghabi *et al*., 2007) and this resistance can be used for the bioremediation of heavy metals. One of the heavy metals that have recently emerged as an important pollutant due to its intense worldwide usage in industries is molybdenum. Its uses include super alloys, nickel base alloys, lubricants, chemicals, glass workings, ink, pigments, electronics and many other applications. It is from these industries that molybdenum can be found in the discharged effluents (Shineldecker, 1992). In the Tokyo Bay and the Black Sea, molybdenum level is in the range of hundreds of ppm (parts per million) making it a significant pollutant (Davis, 1991). Molybdenum pollution in Malaysia have been reported to occur when a ruptured pipe from a molybdenum and copper mining area in Sabah have contaminated of 2000 acres of paddy lands and the Ranau River (Yong, 2000). Scheduled wastes from the above industries are other possible sources (DOE, 2007). The illegal discharged and dumping of the wastes (Mokhtar *et al.*, 2003) have probably contributed towards several reports on heavy metal pollutions in Malaysia (Shukor *et al.*, 2006; Shukor *et al.*, 2008a; Yin *et al.*, 2007). Molybdenum remediation has been recorded in one of the worst molybdenum polluted site in Tyrol, Austria. Molybdenum from industrial exhaust gas has contaminated several hundreds acres of agriculture land causing scouring in cows. Bioremediation using a combination of phytoremediation and bacterial-based remediation was successful in lowering toxic soluble molybdenum content (Neunhäuserer, 2001). Molybdate reduction to Mo-blue by bacteria was first reported by Capaldi and Proskauer more than one hundred years ago in 1896. This is followed by Campbell *et al.* (1985), Sugio *et al.* (1988) and Ghani *et al.* (1993). The first detailed studies on molybdate reduction to Mo-blue were carried out in 1985 by Campbell *et al.*, 1985 in *Eschericia coli* K12. Ghani *et al.* (1993)

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quickly realized the importance of this microbe for the bioremediation of molybdenum. Despite the potential problems of chemical reduction of molybdenum by ferrous irons in the previous works of Sugio *et al.* (1988) and Yong *et al.* (1997), it was later proven that the molybdate reduction in the heterotrophic bacterium, *Enterobacter cloacae* strain 48 is enzymatically mediated (Shukor *et al.*, 2002). We have previously isolated and characterized the molybdenum-reducing *Serratia marcescens* strain Dr.Y6 (Shukor *et al.*, 2008b). This is a second species from the genus *Serratia* with the ability to reduce molybdenum to Mo-blue.

The ongoing efforts to develop a more cost effective strategy in the bioremediation of heavy metals are reflected in the works on chromate-reducing bacteria. Numerous chromate-reducing bacteria were reported over the last thirty years as a continuing effort to isolate more potent reducers (Lloyd, 2003). With this strategy in mind, our screening works have resulted in the isolation of a bacterium that could produce more Mo-blue from 10 mM molybdate than the previously reported heterotrophic molybdenum-reducing bacteria. The characteristics of this bacterium suggest that it would be an important organism for the bioremediation of molybdenum in the future.

Experimental

Materials and Methods

Isolation of molybdenum-reducing bacterium. Soil samples were taken (5 cm deep from topsoil) from a scrap metal recycling ground in Parit, State of Perak, Malaysia in December 2002. Five grams of a well-mixed soil sample were suspended in 45 ml of 9 g/l saline solution. A suitable serial dilution aliquot (0.1 ml) of soil suspension was spread plated onto an agar of low phosphate molybdate (LPM) media (pH 7.0) containing (g/l); glucose (10 g), $(NH_4)_2SO_4$ (3 g), $MgSO_4 \times 7H_2O$ (0.5 g), NaCl (5 g), yeast extract (0.5 g), $Na_2MoO_4 \times 2H_2O$ (2.42 g) and Na_2HPO_4 (0.5 g). Glucose was autoclaved separately. For growth in liquid media we utilized the same medium as described above, omitting solidifying agent. Mo-blue is produced in this medium but not at high phosphate medium (100 mM phosphate). Several white and blue colonies appeared after overnight incubation at room temperature. Blue colonies indicate molybdate reduction activity. One single colony exhibiting the strongest blue intensity visible by naked eye was inoculated into 50 ml of low phosphate media and incubated at 30°C for 24 hours. This bacterium was kept in the department's bacterial culture collection in the Bioremediation and Bioassay Laboratory (Lab 204). The production of Mo-blue from the media was measured at 865 nm. Identification at species level was performed by using Biolog GN MicroPlate (Biolog, Hayward, CA, USA) according to the manufacturer's instructions and molecular phylogenetics studies. *Enterobacter cloacae* strain 48, originally isolated from the town of Chengkau, state of Negeri Sembilan, Malaysia and *S. marcescens* strain Dr.Y6 was obtained from our culture collection. *Enterobacter cloacae* strain 48, *S. marcescens* strain Dr.Y6 and *E. coli* K12 (Sigma) were grown and maintained on the above low phosphate liquid and solid media. For comparing molybdenum-reducing property, one single colony from each bacterium was inoculated into 50 ml of low phosphate media and incubated at 30°C for 24 hours. In order to obtain the Mo-blue spectrum from strain Dr.Y9, a portion of the growth media containing Mo-blue was centrifuged and the supernatant was scanned from 400 to 980 nm (Cintra 5) with freshly prepared low phosphate media as the baseline correction.

Assay for molybdenum-reducing enzyme. The molybdenum-reducing enzyme assay initially uses molybdate as the electron acceptor (Ghani *et al.*, 1993). In further studies, molybdate was changed to 12-phosphomolybdate ($H_3Mo_{12}O_{40}P \times H_2O$, Sigma) (Shukor *et al.*, 2003). More recently we discovered that laboratory-prepared phosphomolybdate gave much higher activity than the commercial 12-phosphomolybdate (Shukor *et al.*, 2008c). Briefly, laboratory-prepared ten to four phosphomolybdate or 10:4 ratios of phosphomolybdate was prepared arbitrarily as a 60 mM stock solution in deionized water by mixing 600 mM molybdate ($Na_2MoO_4 \times 2H_2O$) with 240 mM phosphate ($Na_2HPO_4 \times 2H_2O$). Final pH adjustment of phosphomolybdate solution to pH 5.0 was done using 1 M HCl. Into 1 ml of reaction mixture containing 15 mM (final concentration) laboratory-prepared electron acceptor substrates in 50 mM citrate-phosphate buffer pH 5.0 at room temperature, 100 µl of NADH (80 mM stock) was added to a final concentration of 8 mM. Fifty microlitres of crude Mo-reducing enzyme was added to start the reaction. The absorbance increase in one minute was read at the wavelength of 865 nm. One unit of Mo-reducing activity is defined as that amount of enzyme that produces 1 nmole Mo-blue per minute at room temperature. The specific extinction coefficient at 865 nm is 16.7 $mM^{-1} \times cm^{-1}$ (Shukor *et al.*, 2001). An increase in absorbance at 865 nm of 1.00 unit absorbance per minute per mg protein would yield 60 nmole of 12-phoshomolybdate or 60 units of enzyme activity in a 1 ml assay mixture.

Preparation of crude enzyme. Bacteria were grown in one liter of media containing high phosphate at 30°C for 24 hours on an orbital shaker (100 rpm). This is because although the high phosphate inhibited molybdate reduction to Mo-blue, the cells contain active enzymes (Ghani *et al.*, 1993) and growth on low

phosphate resulted in a blue sticky culture that complicated the preparation of crude enzyme and enzyme assay. The following experiment was carried out at 4°C unless stated otherwise. Cells were harvested through centrifugation at 10 000 g for 10 minutes. Cells were washed at least once with distilled water, resuspended and recentrifuged. The pellet was reconstituted with 10 ml of 50 mM Tris buffer pH 7.5 (Tris buffer prepared at 4°C). Cells were sonicated for 1 minute on an ice bath with 4 minutes cooling until a total sonication time or at least 20 minutes was achieved. The sonicated fraction was centrifuged at 10 000 g for 20 minutes and the supernatant consisting of the crude enzyme fraction was taken.

The effects of metabolic inhibitors. Metabolic inhibitors such as HQNO (Hydroxyquinoline-N-Oxide), antimycin A, sodium azide, potassium cyanide and rotenone were prepared in either deionised water or acetone and added into enzyme assay mixture in a volume not exceeding 20% of assay volume to prevent shifting in assay pH. One hundred microlitres of enzyme was added into the assay mixture to start the reaction. Deionised water was added so that the total reaction mixture was 1 ml. As a control, 50 µl of acetone was added in the reaction mixture without inhibitors. The increase in absorbance at 865 nm was measured after a period of 5 minutes.

16S rDNA gene sequencing and phylogenetic analysis. Genomic DNA was extracted from bacterial colonies by alkaline lysis. PCR amplification was performed using a Biometra T Gradient PCR (Montreal Biotech Inc., Kirkland, QC). The PCR mixture contained 0.5 pM of each primer, 200 mM of each deoxynucleotide triphosphate,1x reaction buffer, 2.5 U of Taq DNA polymerase (Promega) to achieve a final volume of 50 m1. The 16S rDNA gene from the genomic DNA was amplified by PCR using the following primers; 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3' corresponding to the forward and reverse primers of 16S rDNA, respectively (Devereux and Wilkinson, 2004). PCR was performed under the following conditions: initial denaturation at 94°C for 3 min; 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 10 min. Cycle sequencing was subsequently performed with the Big Dye terminator kit (Perkin-Elmer Applied Biosystems) as recommended by the manufacturer. Sequence data were initially recorded and edited using CHROMAS Version 1.45. The resultant 1452 bases were compared with the GenBank database using the Blast server at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). This analysis showed this sequence to be closely related to *rrs* from Gammaproteobacteria. The 16S rRNA ribosomal gene sequence for this isolate have been deposited in GenBank under DQ226210 accession number.

Phylogenetic analysis. A multiple alignment of 18 16S rRNA gene sequences closely matches strain Dr.Y9 were retrieved from GenBank and were aligned using ClustalW (Thompson *et al.*, 1994) with the PHYLIP output option. A phylogenetic tree was constructed by using PHYLIP, version 3.573 (J.Q. Felsenstein, PHYLIP-phylogeny inference package, version 3.573, Department of Genetics, University of Washington, Seattle, WA. (http://evolution.genetics.washington.edu/phylip.html), with *Bacillus subtilis* as outgroup in the phylogram. Evolutionary distance matrices for the neighbour-joining/UPGMA method were computed using the DNADIST algorithm program (Jukes and Cantor, 1969). Phylogenetic tree (Fig. 1) was inferred by using the neighbour-joining method of Saitou and Nei (1987). With each algorithm, confidence levels for individual branches within the tree were checked by repeating the PHYLIP analysis with 1000 bootstraps (Felsenstein, 1985) by the SEQBOOT program in the PHYLIP package. Majority rule (50%) consensus trees were constructed for the topologies found using a family of consensus tree methods called the Ml methods (Margush and McMorris, 1981) using the CONSENSE program and the tree was viewed using TreeView (Page, 1996).

Statistical analysis. Values are means ± SE. All data were analyzed using Graphpad Prism version 3.0 and Graphpad InStat version 3.05. Comparison between groups was performed using a Student's t-test or a one-way analysis of variance with post hoc analysis by Tukey's test. $P<0.05$ was considered statistically significant.

Results and Discussions

Identification of the isolate. A moderate bootstrap value (>50%) was seen when strain Dr.Y9 is associated to clades harboring several *S. marcescens* strains *indicating* that the phylogenetic identity of this bacterium is *S. marcescens* (Fig. 1). The identifications performed by Biolog GN also give conclusive identification to *S. marcescens* with high probability (99.9%) values. Thus, this bacterium is assigned tentatively as *S. marcescens* strain Dr.Y9. Previously we have isolated a different *S. marcescens* (Shukor *et al.*, 2008b). In an earlier works of Jan (1939), another genus of *Serratia* sp. was reported to be able to reduce molybdate to Mo-blue. However comparison could not be made since there were no standard methods reported to quantify or assay the Mo-blue produced. Table I showed the amount of Mo-blue from a 24-hours culture of *Serratia* sp. strain Dr.Y9, *E. cloacae* strain 48 and *E. coli* K12. ANOVA shows that isolate Dr.Y9 produced significantly higher ($p<0.05$) amount of Mo-blue with 2.83, 5.1 and 9.6 times more

Table I
Amount of Mo-blue produced from a 24-hours culture of strain Dr.Y9. Data are given as the mean values between three determinations. Values with the same letter (b and c) are not significantly different (p>0.05)

Bacterial strains	Micromole Mo blue produced after 24 hours of static incubation
S. marcescens strain Dr.Y9	9.91 ± 0.43^{a}
S. marcescens strain Dr.Y6	3.49 ± 0.14^{b}
E. cloacae strain 48	1.94 ± 0.52^{bc}
E. coli K12	1.03 ± 0.11^{c}

Mo-blue than *S. marcescens* strain Dr.Y6, *E. coli* K12 and *E. cloacae* strain 48, respectively. Molybdate reduction in this bacterium was optimum at 37°C and pH 6.0 (data not shown).

The effect of electron donor. In the presence of 10 mM molybdate, only glucose, sucrose, fructose, mannitol, and starch (at an initial concentration of 2 g/l) supported molybdate reduction with sucrose supporting the significantly highest amount of Mo-blue compared to the rest of the electron donors ($p<0.05$) (Fig. 2). Precipitation of bluish cellular mass was observed during the reduction of molybdate and repeated centrifugation and resuspension of the cellular mass with several volumes of deionised buffer showed that it was impossible to remove the reduced product. This prevented measurements of growth kinetics to be carried out. Interestingly, a similar observation is seen in *E. cloacae* strain 48 but not reported in *E. coli* K12. Ghani *et al.* (1993) also reported that sucrose gives the highest rate of molybdate reduction. Campbell *et al.* (1985) reported that the best electron donor that could support molybdate reduction in *E. coli* K12 is glucose, although sucrose was not one of the electron donors tested whilst formate, succinate, glycerol and ethanol did not support reduction. In *S. marcescens* strain Dr.Y6, sucrose was the best electron donor followed by maltose, glucose and glycerol. Other electron donors such as acetate, formate, citric acid, lactose, mannitol, tartarate, maltose, and starch did not support reduction. Optimum concentration of sucrose for molybdate reduction was 10 g/l (data not shown).

The effects of phosphate and molybdate concentrations on molybdate reduction. When phosphate concentration was fixed at 2.9 mM, the optimal

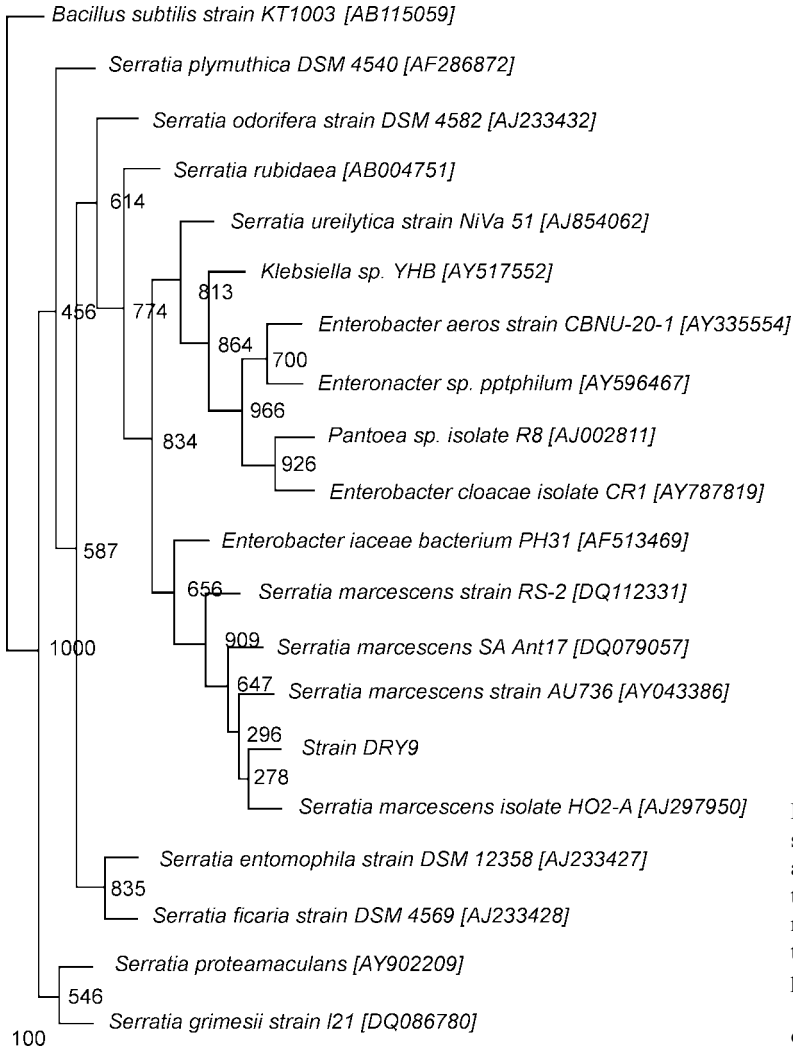

Fig. 1. A phylogram (neighbour-joining method) showing genetic relationship between strain Dr.Y9 and other related reference microorganisms based on the 16S rRNA gene sequence analysis. Species names are followed by the accession numbers of their 16S rRNA sequences. The numbers at branching points or nodes refer to bootstrap values, based on 1000 re-samplings. Scale bar represents 100 nucleotide substitutions. *B. subtilis* is the outgroup.

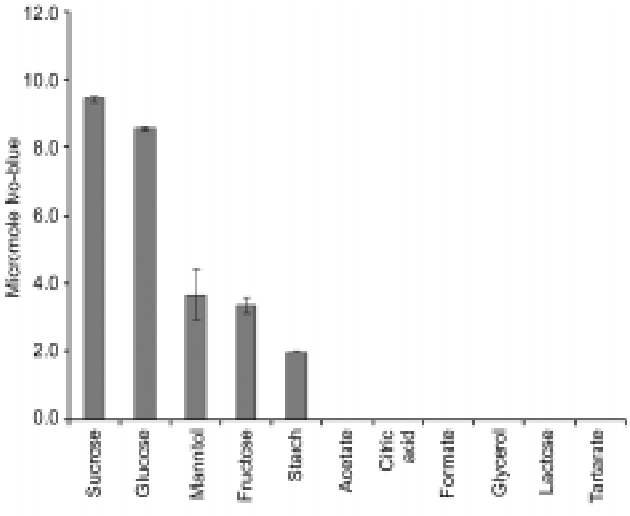

Fig. 2. Molybdate reduction using various electron donors by strain Dr.Y9. Molybdate reduction is considered negligible if the absorbance at 865 nm is below 0.020. Error bars represent mean ± standard error (n = 3).

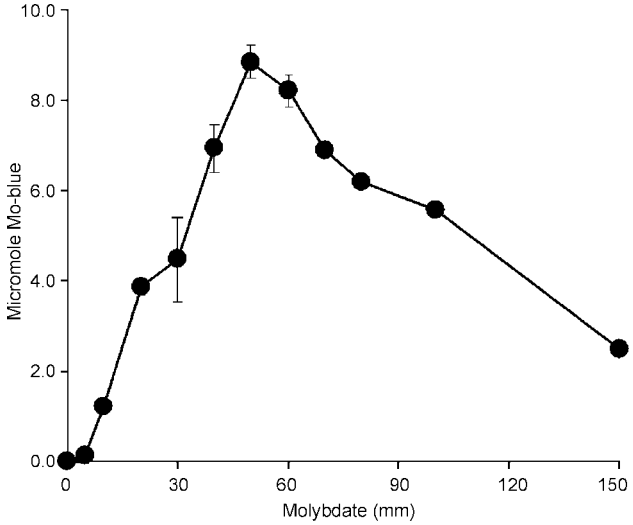

Fig. 3. The effect of molybdate concentrations on molybdate reduction by strain Dr.Y9. Error bars represent mean ± standard error (n = 3).

range of molybdate concentration that supported molybdate reduction was from 50 to 60 mM of molybdate and reduction to Mo-blue was inhibited at higher molybdate concentrations. The bacterium was able to tolerate and reduce molybdate to Mo-blue even at concentrations of molybdate as high as 150 mM of molybdate indicating good tolerance towards molybdenum (Fig. 3). In comparison, *S. marcescens* strain Dr.Y6 has an optimum molybdate reduction between 15 and 25 mM molybdate (at 5 mM phosphate) and higher molybdate concentrations strongly inhibited molybdate reduction (Shukor *et al.*, 2008b). In *E. coli* K12, molybdate reduction occurs optimally at 80 mM molybdate but the phosphate concentration must be fixed at 5 mM (Campbell *et al*., 1985). In order to know the optimal phosphate concentration of phosphate that supported reduction, we fixed the molybdate concentration at 55 mM. We discovered that the optimum concentration of phosphate was 5 mM and molybdate reduction dramatically decreased at much higher phosphate concentrations. More than 60% of molybdate reduction activity was lost at phosphate concentrations higher than 20 mM and reduction was totally inhibited at 100 mM phosphate (Fig. 4). Ghani *et al.* (1993) reported that phosphate concentration greater than 0.5 mM inhibits molybdate reduction in *E. cloacae* strain 48. On the contrary to *E. cloacae* strain 48, all of the other bacteria reported so far including in this work required 5 mM of phosphate for optimal molybdate reduction to Mo-blue. High phosphate concentration probably inhibits molybdate reduction by maintaining the pH at neutral; a pH that is undesirable for the formation, and stability of phosphomolybdate (Glenn and crane, 1956; Lee, 1977; Shukor *et al.*, 2001).

The absorption spectra of Mo-blue. As molybdate reduction progresses, there is an increase in an overall absorption profiles especially at the peak maximum at 865 nm and the shoulder at 700 nm concomitant with the increasing blue intensity of the media (Fig. 5). The spectrum of Mo-blue produced by this isolate is closely similar to the Mo-blue product of *E. cloacae* strain 48 with a peak maximum at 865 nm and a shoulder at 700 nm (Shukor *et al*., 2000). Recently, we have also isolated several molybdenum-reducing strains all with Mo-blue spectra highly similar to this works (Shukor *et al*., 2007). The spectra are distinctly not similar to the absorption spectra of other Mo-blue products such as silicomolybdate and sulfomolybdate (Yoshimura *et al.*, 1986; Hori *et al.*, 1988; Shukor *et al.*, 2008d). These strains are currently being characterized. We notice that the

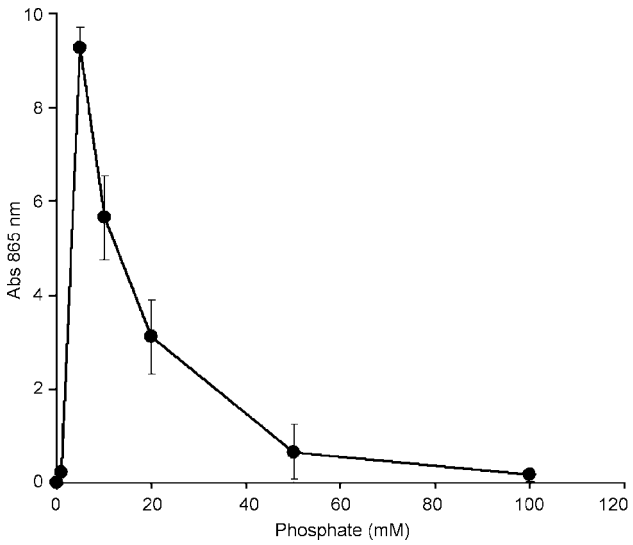

Fig. 4. The effect of phosphate concentrations in the media on molybdate reduction by strain Dr.Y9. Error bars represent mean ± standard error (n = 3).

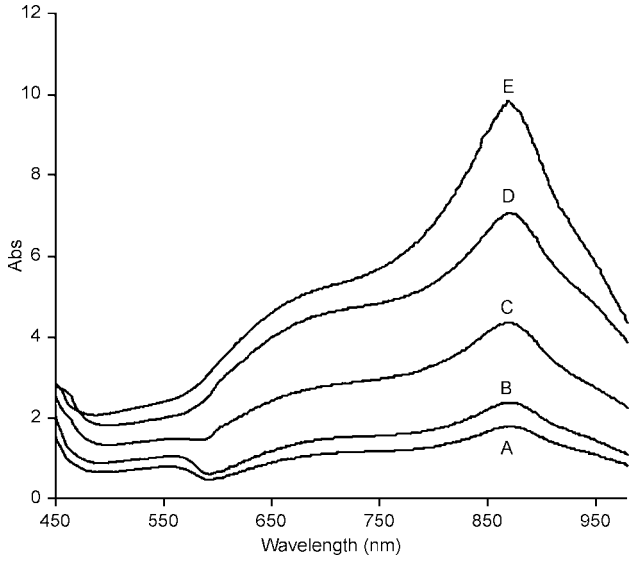

Fig. 5. Scanning spectra of Mo-blue after 14, 16, 18, 20 and 24 hours of static incubation labelled A, B, C, D and E, respectively.

spectrum is similar to the Mo-blue produced by the ascorbic acid-reduced phosphomolybdate in the phosphate determination method (Clesceri *et al.*, 1989). In the heteropolyblue method, determination of phosphate concentration was measured at 880 nm using ascorbic acid as the reducing agent. This suggests that the increase at 865 nm observed in the absorption spectra during molybdate reduction by strain Dr.Y9 was likely an increase in the reduction of phosphomolybdate. Identification of reduced phosphomolybdate based on spectroscopy is generally enough to distinguish between one heteropolymolybdate to another *e.g.* between phosphomolybdate and silicomolybdate or sulfomolybdate (Glenn and Crane, 1956; Sims, 1961; Kazansky and Fedotov, 1980; Yoshimura *et al.*, 1986; Hori *et al.*, 1988).

The effects of metabolic inhibitors. Proposed site of Mo-reduction in *E. cloacae* strain 48 was suggested to be component(s) after the cytochrome b complex and before cytochrome o/d oxidase (Ghani et *et al.*, 1993). However the substrate for molybdate reduction assay for *E. cloacae* strain 48 is different since molybdate was used at the electron acceptor, while in this work phosphomolybdate was used instead. The results in Table II showed that all of the inhibitors tested did not show any inhibition to Mo-reducing activity in this bacterium ($F_{6,14} = 0.1983$, P>0.05). Antimycin A and HQNO are inhibitors to cytochrome b. Rotenone is an inhibitor to NADH dehydrogenase while sodium azide and cyanide are inhibitors to the terminal cytochrome d oxidase (Dawson, 1969). Since the new enzyme assay utilizes phosphomolybdate instead of molybdate as in the original assay (Ghani *et al.*, 1993), we repeated the experiment using molybdate as the electron accepting substrate. Interestingly, we obtained similar results with no inhibition of molybdenum-reducing activity seen (data not shown). The results suggest that the electron transport system of this bacterium is not the site of molybdate reduction. In contrast, the ETS has been previously suggested as the site of molybdate reduction in *E. cloacae* strain 48 (Ghani *et al.*, 1993) based on the inhibition of Mo-reducing enzyme by cyanide. The effects of respiratory inhibitors were however not carried out in *E. coli* K12 (Campbell *et al.*, 1985) and *S. marcescens* strain Dr.Y6 (Shukor *et al.*, 2008b).

Table II
Effect of respiratory inhibitors on molybdate reduction

Inhibitors (Concentration added into reaction mixture)	Concentration which normally gives 50% inhibition per mg protein*	% Mo-blue Activity
HQNO	10^{-8} M to 10^{-5} M	98.53 ± 1.76
Rotenone	10^{-8} M	81.50 ± 5.81
Azide	1 mM	82.60 ± 10.73
Cyanide	~ 0.5 mM	102.41 ± 8.81
Antimycin	0.5 uM	115.71 ± 7.35
Control	–	102 ± 1.14

* Dawson, (1969)

Conclusions. We have isolated a local molybdenum-reducing bacterium studied the effect of various parameters such as nitrogen and electron donors, temperature, molybdate and phosphate on molybdate reduction from this bacterium. The optimum conditions for molybdate reduction were similar to previously studied heterotrophic molybdenum-reducing bacteria. Due to the limited literature on the variety of molybdenum-reducing bacterium, our works sought to increase the repertoire of bacterial species able to reduce molybdate to Mo-blue. Knowledge of the various optimized parameters would facilitate an easy and more effective translation of the laboratory results to the fields. Currently, work is underway to purify the molybdenum-reducing enzyme from this bacterium. We are also currently isolating and characterizing more molybdenum-reducing bacterium to screen for multi-metal resistant strains.

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