Research Article

Kinetics of Molybdenum Reduction to Molybdenum Blue by Bacillus sp. Strain A.rzi

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Received 14 September 2013; Accepted 28 October 2013

Academic Editor: Kannan Pakshirajan

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Molybdenum is very toxic to agricultural animals. Mo-reducing bacterium can be used to immobilize soluble molybdenum to insoluble forms, reducing its toxicity in the process. In this work the isolation of a novel molybdate-reducing Gram positive bacterium tentatively identified as Bacillus sp. strain A.rzi from a metal-contaminated soil is reported. The cellular reduction of molybdate to molybdenum blue occurred optimally at 4 mM phosphate, using 1% (w/v) glucose, 50 mM molybdate, between 28 and 30°C and at pH 7.3. The spectrum of the Mo-blue product showed a maximum peak at 865 nm and a shoulder at 700 nm. Inhibitors of bacterial electron transport system (ETS) such as rotenone, sodium azide, antimycin A, and potassium cyanide could not inhibit the molybdenum-reducing activity. At 0.1 mM, mercury, copper, cadmium, arsenic, lead, chromium, cobalt, and zinc showed strong inhibition on molybdate reduction by crude enzyme. The best model that fitted the experimental data well was Luong followed by Haldane and Monod. The calculated value for Luong’s constants \( p_{\text{max}} \), \( K_s \), \( S_m \), and \( n \) was 5.88 μmole Mo-blue hr⁻¹, 70.36 mM, 108.22 mM, and 0.74, respectively. The characteristics of this bacterium make it an ideal tool for bioremediation of molybdenum pollution.

1. Introduction

Heavy metals are ubiquitously applied in numerous industrial processes. This has resulted in heavy metals contamination of many environmental systems in Malaysia [1–3]. Molybdenum is an example of a heavy metal with numerous applications in industries. It is a significant pollutant with levels as high as thousands of ppm found in aquatic bodies and soils [4, 5]. The toxicity of molybdenum compounds has been studied intensively in animals [6, 7]. Cows are the most affected with dramatic scouring occurring at 20–50 mg Mo/kg body weight, followed by sheep and pigs [8]. In Malaysia, molybdenum is produced as a byproduct of copper from a mine in Sabah [9].

Like other heavy metals pollution, scientists have turned towards bioremediation, a cheaper alternative using the ability of microbe to remove and resist heavy metals via mechanisms such as sequestration, bioreduction, biosorption, transport mechanisms, bioprecipitation, and/or chelation [10]. Microbial reduction of molybdate (Mo⁶⁺) to Mo-blue was first reported in 1896 [11]. Since the last thirteen years, almost all of the reported bacteria capable of microbiological reduction of molybdate to molybdenum blue [12–25] came from our work. Screening for novel molybdenum reducers is required in order to develop a cost-effective bioremediation work for cleaning up molybdenum pollutants in the environment [26]. Numerous commercial bioremediating bacteria use Bacillus spp. as this genus offers several advantage such as...
extreme environmental tolerance due to its capability to form endospore and a fast doubling time [27]. In this work, we report on the isolation of a novel molybdenum-reducing *Bacillus* sp. strain A.rzi and its kinetics of molybdenum reduction to molybdenum blue (Mo-blue).

2. Experimental

2.1. Molybdenum-Reducing Bacterium Isolation. Soil samples were taken from a metal recycling plant in Kajang, Selangor, in December 2007. Soil sample (five gram) was suspended in 50 mL of phosphate buffered saline (1x). Suitable serial dilutions (aliquot 0.1mL) were spread onto molybdenum selective agar of low phosphate (2.9 mM phosphate) media (pH 7.5) supplemented with glucose (10 g L−1) as the carbon source, NaCl (5 g L−1), (NH4)2SO4 (3 g L−1), Na2MoO4·2H2O (2.42 g L−1), MgSO4·7H2O (0.5 g L−1), yeast extract (0.5 g L−1), and Na2HPO4·2H2O (0.51 g L−1) [17]. The strongest blue colony on the plate was transferred into 50 mL of liquid low phosphate media. Molybdenum-reducing bacterial strains such as *Serratia* sp. strain Dr.Y8, *Serratia* sp. strain Dr.Y5, *S. marcescens* strain Dr.Y9, *Enterobacter* sp. strain Dr.Y13, *Pseudomonas* sp. strain DRY2, *Serratia marcescens* strain DRY6, *Acinetobacter calcoaceticus* strain Dr.Y12, and *Enterobacter cloacae* strain 48 were obtained from our culture collection and *E. coli* K12 was obtained from American Type Culture Collection, Rockville, USA. The bacteria were grown and maintained on the above low phosphate liquid and solid media, respectively.

2.2. Molybdenum-Reducing Bacterium Identification. Identification of the bacterium was performed by using Biolog GP microplate (Biolog, Hayward, CA, USA) according to the manufacturer’s instructions and molecular phylogenetic studies. Genomic DNA was prepared through alkaline lysis method. PCR amplification was carried out using a Biometra T Gradient PCR (Montreal Biotech Inc., Kirkland, QC). The PCR mixture comprises 0.5 μM of the following primers: 5’-AGAGTTTGTATCTGCTCAG-3’ and 5’-AAGGAGGTGATCCAGCGCAAC-3’ corresponding to the forward and reverse primers of 16S rDNA, respectively [28], 2.5 U of Taq DNA polymerase (Promega), 200 μM of each deoxynucleotide triphosphate, and 1x reaction buffer. The reaction mixture had a final volume of 50 μL. The 16S rDNA gene from the genomic DNA was amplified by PCR using the following conditions: an initial denaturation at 94°C for 3 min; 25 cycles at 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. The Big Dye terminator kit (Perkin-Elmer Applied Biosystems) was used for cycle sequencing. The resultant 1306 bases were compared to similar sequence in the GenBank database using the NCBI Blast server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The 16s rRNA ribosomal gene sequence was deposited in GenBank under the accession number EU835195.

2.3. Phylogenetic Analysis. Alignment of 20 16S rRNA gene sequences closely matching strain A.rzi retrieved from GenBank was carried out using clustal_W [29]. The construction of the phylogenetic tree was carried out 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)). *S. marcescens* was the outgroup in the cladogram. The neighbour-joining/UPGMA method was used to construct the evolutionary distance matrices the DNADIST algorithm program. A distance matrix was used instead of the laborious maximum likelihood or parsimony approaches. The model used in the nucleotide substitution is from Jukes and Cantor [30]. Phylogenetic tree was constructed based on the neighbour-joining method adopted from Saitou and Nei [31]. Confidence levels for individual branches within the tree were checked for each algorithm by repeating the PHYLIP analysis with 1000 bootstraps [32] using the SEQBOOT program in the PHYLIP package. The jackknife approach can also be used. Majority rule (50%) consensus trees were constructed using the Ml methods [33]. The tree was viewed using the TreeView program [34].

2.4. Crude Enzyme Preparation. The preparation of crude enzyme was based on the modified method of Shukor et al. [35]. Experiments were carried out at 4°C unless stated otherwise. A 2 L culture was grown overnight on high phosphate media (HPM) containing MgSO4·7H2O (0.5 g L−1), NaCl (5 g L−1), (NH4)2SO4 (3 g L−1), Na2MoO4·2H2O (12.1 g L−1 or 50 mM), yeast extract (1 g L−1), glucose (10 g L−1) as an electron donor source, and Na2HPO4 (100 mM) at pH 7.3. The bacterial cells were first harvested at 10000 × g for 20 min at 4°C. The pellet was washed several times and reconstituted with 10 mL of 50 mM Tris buffer (pH 7.0) containing 1 mM phenylmethanesulphonyl fluoride (PMSF) as a protease inhibitor and 2 mM of DTT as a reducing agent for protecting the thiol group in the enzyme. The cells were then subjected to sonication on a Biosonik III sonicator for a total sonication time of 2 hours with intermittent cooling on an ice bath and then ultracentrifuged at 105000 × g for 90 min at 4°C. The supernatant was the crude enzyme and was used for further studies. Enzyme was assayed according to the method of Shukor et al. [35] by adding 100 μL of NADH (80 mM stock) 1 mL of a reaction mixture consisting of laboratory-prepared phosphomolybate (LPP) electron acceptor substrates prepared in 50 mM citrate-phosphate buffer pH 5.0 at room temperature. The final concentration of LPP was 8 mM. To start the reaction, fifty microlitres of crude Mo-reducing enzyme were added. The absorbance increase in a one minute incubation period was read at 865 nm. The definition of one unit of Mo-reducing activity is the amount of enzyme that produces 1 nmole of Mo-blue per minute at room temperature. The specific extinction coefficient for the product Mo-blue at 865 nm is 16.7 mM−1·cm−1 [36].

2.5. Studies on the Effects of Metal Ions and Respiratory Inhibitors. Inhibitors such as sodium azide, antimycin A, rotenone, and potassium cyanide were dissolved in deionised water and/or in acetone [37]. Metal ions were dissolved in 20 mM Tris-Cl buffer (pH 7.0). A preincubation of the inhibitors or metal ions with one hundred microlitres of enzyme in
the reaction mixture was carried out at 4°C for 10 minutes. The incubation mixture was warmed to room temperature before NADH was added to start the reaction. The total reaction mixture was 1.0 mL. As a control for inhibitors that was dissolved in acetone such as rotenone and antimycin A, 50 μL of acetone was added in the reaction mixture without inhibitors. The linear increase in absorbance at 865 nm was measured after an incubation period of 5 minutes.

2.6. Characterization of the Molybdate Reduction Reaction Using the Dialysis Tubing Method. The dialysis tubing method is a modification of the method applied by Hem [41] to identify whether the reduction of heavy metals seen physiologically was due to biotic chemicals produced by the cells or catalyzed through an enzymatic route. The dialysis tubing method of Shukor et al. [23] was used in this work.

2.7. Determination of Kinetic Parameters for Molybdate Reduction to Molybdenum Blue. Determination of intrinsic growth kinetic parameters for strain A.rzi was not possible due to the property of the molybdenum blue that form a precipitate together with the bacterial mass [18–25]. Hence, only the reduction kinetics was studied. Several substrate inhibition kinetic models available such as Haldane and Luong were compared to the commonly used Monod. In this work molybdenum reduction kinetics is represented as Mo-blue production rate. The formula for the above model is shown in Table 1, where \( p, p_{\text{max}}, K_s, K_i, S, S_m, \) and \( n \) are specific Mo-blue production rate (hr\(^{-1}\)), maximum Mo-blue production rate (hr\(^{-1}\)), half-saturation constant (mM), inhibition constant (mM), substrate concentration (mM), critical substrate concentration above which production of Mo-blue completely stops (mM), and the exponent representing the impact of the substrate to \( p_{\text{max}} \), respectively. The Mo-blue production rate is calculated based on the linear portion of the Mo-blue production against time.

2.8. Statistical Analysis. Comparison between groups was performed using a Student’s t-test or a one-way analysis of variance (ANOVA) with post hoc analysis by Tukey’s test. \( P < 0.05 \) was considered statistically significant.

3. Results

3.1. Identification of Mo-Reducing Bacterium. A Gram-positive spore-forming bacterium capable of molybdenum reduction to molybdenum blue was isolated from a metal-contaminated soil. The bacterium was identified through phylogenetic analyses of the 16S rRNA ribosomal gene sequence of the bacterium. A high bootstrap value (>75%) of 79.8% was obtained when strain A.rzi is genetically linked to Bacillus sp. (Figure 1). This is a novel molybdenum-reducing Gram positive bacterium (Figure 1). The identifications performed using GP2 plate with the BIOLOG system gave 98% probability, 0.747 similarity, and 3.56 distance value to Bacillus pumilus B. At this juncture, we tentatively assigned this bacterium as Bacillus sp. strain A.rzi.

3.2. Comparison of Mo-Blue Production among Mo-Reducing Isolates. When grown in 10 mM molybdate as the benchmark molybdate concentration, strain A.rzi ranked as the second best together with Serratia sp. strain Dr.Y5 in producing Mo-blue (Table 2). The optimal carbon sources of either sucrose or glucose supporting molybdate reduction for each bacterium [18–25] were used in this comparison work. Mo-blue production increases dramatically after 18 hours of static growth, reaching maximal production after 23 hours of incubation (Figure 2).

3.3. The Effects of Phosphate and Molybdate Concentrations. The effect of molybdate concentration on molybdate reduction (Figure 3) showed that the optimum concentration of molybdate was between 50 and 60 mM. Concentrations higher than 80 mM molybdate were strongly inhibitory. At 50 mM molybdate, molybdate reduction was strongly critical of phosphate concentration with a sharp optimum at 4 mM and a near complete inhibition of Mo-reduction at higher phosphate concentrations (Figure 4).

3.4. The Effect of pH and Temperature on Molybdate Reduction. The optimum temperature supporting molybdate reduction was in the range from 28 to 30°C (Figure 5). The effect of pH was carried out using 50 mM of Tris and 10 mM of phosphate buffers spanning the pH from 7.0 to 9.0. The optimum pH for molybdenum reduction is pH 7.3 (data not shown).

3.5. The Effect of Electron Donor Sources on Molybdate Reduction. Glucose was the most effective supplement as an electron donor for supporting molybdate reduction. This was followed by sucrose, maltose, mannose, mannitol, lactose, and starch (Figure 6). Glucose was optimum at 1% (w/v).

3.6. Mo-Blue Absorbance Spectrum. The spectrum of Mo-blue obtained from the growth medium shows a maximum peak near the far red region between 860 and 870 nm and a shoulder approximately at 710 nm (Figure 7).
Figure 1: Phylogram (neighbour-joining method) indicating the 16s rRNA genetic relationship between 20 other related references microorganisms from the GenBank database and strain A.rzi. *S. marcescens* is the outgroup. Species names of bacteria were followed by the accession numbers of 16s rRNA. The internal labels at the branching points are the bootstrap value. Scale bar represents 100 nucleotides substitution.

| Table 2: Amount of Mo-blue produced by a 24-hour static culture of strain A.rzi. Values are mean ± standard error (n = 3). |
|---------------------------------------------------------------|
| **Bacteria** | **Micromole Mo-blue** |
| Bacillus sp. strain A.rzi | 7.82 ± 0.24<sup>a</sup> |
| Serratia sp. strain Dr.Y8 | 10.41 ± 0.13<sup>b</sup> |
| S. marcescens strain Dr.Y9 | 9.86 ± 0.44<sup>b</sup> |
| Serratia sp. strain Dr.Y5 | 7.87 ± 0.15<sup>a</sup> |
| Pseudomonas sp. strain DRY2 | 6.94 ± 0.65<sup>c</sup> |
| Enterobacter sp. strain Dr.Y13 | 6.91 ± 0.15<sup>c</sup> |
| Acinetobacter calcoaceticus strain Dr.Y12 | 5.86 ± 0.14<sup>d</sup> |
| Serratia marcescens strain DRY6 | 2.84 ± 0.23<sup>e</sup> |
| Enterobacter cloacae strain 48 | 2.17 ± 0.56<sup>e</sup> |
| Escherichia coli K12 | 0.96 ± 0.04<sup>f</sup> |

Value with the same letter is not significantly different (P > 0.05).

3.7. Studies on the Effects of Metal Ions and Respiratory Inhibitors. Preliminary results indicated that stannous and ferrous ions resulted in a chemical reduction of phosphomolybdate to Mo-blue in the reaction mixture. Hence these metal ions were omitted from this study. At 0.1 mM, mercury, copper, cadmium, arsenic, lead, chromium, cobalt, and zinc showed strong inhibition on molybdate reduction by crude enzyme (Figure 8).

It was found that the inhibitors antimycin A, potassium cyanide, sodium azide, and rotenone did not inhibit more than 10% of the Mo-reducing activity in strain A.rzi (data not shown).
3.8. Characterization of the Molybdate Reduction Reaction Using the Dialysis Tubing Method. The results showed that 95% of the amount of Mo-blue formed (13.775 μmole) was found in the dialysis tube, while only 5% (1.225 μmole) was found at the outside of the tube.

3.9. Kinetics of Molybdenum Blue Production. Data from the experimental value in batch studies was fitted to several kinetic models of growth or product formation, that is, Monod, Luong, and Haldane. CurveExpert Professional software (Version 1.6) with custom equation algorithm that leads to the minimization of the sums of square of residuals was used to find the constants. The best model that fitted the experimental data well was Luong followed by Haldane and Monod with correlation coefficient values of 0.99, 0.83, and 0.36, respectively (Figure 9). The calculated value for $P_{\text{max}}$, $K_s$, $S_m$, and $n$ was 5.88 μmole Mo-blue $\text{hr}^{-1}$, 70.36 mM, 108.22 mM, and 0.74, respectively.

4. Discussions

In the last decade, works on microbial molybdenum reduction to Mo-blue have been restricted to our isolates [18–25, 35, 36, 42]. The enzyme responsible for the reduction has never been purified. Despite this, novel enzyme assay for this enzyme has been developed [35] and purification of this enzyme is being intensely pursued. Our quest for more variety of Mo-reducing bacteria to suit a plethora of environmental conditions has led us to the discovery of this
bacterium. Molybdenum bioremediation can take advantage of this genus ability to produce spores that can be stored for long period and highly resistant to environmental stresses including heavy metals [43].

This bacterium was able to produce comparable molybdenum to other previously isolated strains. Furthermore it is able to reduce a high initial concentration of sodium molybdate of 80 mM. Average reduction at moderate starting molybdate concentrations from previous studies ranges from 25 to 55 mM [17–25]. Tolerance and reduction at concentrations higher than 20 mM are an advantage to a microbe as molybdenum pollution could reach as high as 2000 ppm (20.8 mM molybdate) [44], a concentration lethal to ruminant [45].

The optimum ratio of phosphate to molybdate concentrations is important for overall molybdenum reduction to molybdenum blue. The results obtained in this work show some similarity to the results obtained from Serratia sp. strain Dr.Y8 [19] and S. marcescens strain Dr.Y9 [21]. Other strains show a lower requirement for molybdenum of between 15 and 50 mM but with a similar optimal phosphate at 5 mM [18, 20, 22–25]. The inhibitory effect shown by high phosphate concentrations is probably due to physical interaction with the phosphomolybdate substrate and not through inhibiting the enzymatic action. This has been discussed in detail in other similar publications [18–25]. The Mo-blue spectrum obtained in this strain is similar to all of the other Mo-reducing strains.
such as mercury [54], arsenate [55], and chromate [56]
concentration of molybdenum that completely inhibited Mo-
high concentration of molybdenum with a calculated critical
a clear strong inhibition of Mo-blue production rate at
the Mo-blue formed.
outside of the tubing is almost all due to slow leakage of
exclusively enzymatic in origin as the 5% of Mo-blue found at
the dialysistubing experiment suggeststhat Mo-reduction is
the ET C [52]
report Haldane model [39], the Luong model allows for
the determination of the critical concentration of substrate
that could completely inhibit production of product [40] as
evident from this work. This is the first time existing models
of kinetic studies being applied to model Mo-blue production
in bacterium.

To conclude, we reported on the isolation of a novel molybdenum reducing bacterium from the Bacillus genus.
features of this bacterium, such as temperature, pH, and con-
centration of phosphate that supported the optimal reduc-
tion of molybdenum blue, were similar to other reported
molybdenum-reducing bacterial species. The absorption
spectrum of the Mo-blue product was very similar to other
Mo-reducing bacteria isolated to date indicating probably
the same phosphomolybdate species involved in bacterial reduc-
tion process. The reduction of molybdenum to Mo-blue is
predominantly enzymatic as evident from the dialysis tub-
ing experiment. This bacterium is sensitive towards heavy
metals as was similarly discovered in previously isolated
molybdenum-reducing bacteria and could pose a problem if
the bioremediation site is cocontaminated with other toxic
heavy metals. We also showed for the first time that the Luong
model of substrate (molybdate) inhibition kinetics of Mo-
blue production was better than the Haldane model. We are
currently focusing on the isolation of metal-resistant Mo-
reducing bacteria and the purification of the Mo-reducing
enzyme.

Conflict of Interests
The authors declare that there is no conflict of interests
regarding the publication of this paper.

Acknowledgment
This project was supported by funds from the Research
University Grant Scheme (RUGS), Project no. 05-01-09-
0750RU/F1.

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