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Hexavalent Molybdenum Reduction to Molybdenum Blue by *S. Marcescens* Strain Dr. Y6

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Abstract A molybdate-reducing bacterium has been locally isolated. The bacterium reduces molybdate or Mo^{6+} to molybdenum blue (molybdate oxidation states of between 5+ and 6+). Different carbon sources such as acetate, formate, glycerol, citric acid, lactose, fructose, glucose, mannitol, tartarate, maltose, sucrose, and starch were used at an initial concentration of 0.2% (*w*/*v*) in low phosphate media to study their effect on the molybdate reduction efficiency of bacterium. All of the carbon sources supported cellular growth, but only sucrose, maltose, glucose, and glycerol (in decreasing order) supported molybdate reduction after 24 h of incubation. Optimum concentration of sucrose for molybdate reduction is 1.0% (*w*/*v*) after 24 h of static incubation. Ammonium sulfate, ammonium chloride, valine, OH-proline, glutamic acid, and alanine (in the order of decreasing efficiency) supported molybdate reduction with ammonium sulfate giving the highest amount of molybdenum blue after 24 h of incubation at 0.3% (*w*/*v*). The optimum molybdate concentration that supports molybdate reduction is between 15 and 25 mM. Molybdate reduction is optimum at 35 °C. Phosphate at concentrations higher than 5 mM strongly inhibits molybdate reduction. The molybdenum blue produced from cellular reduction exhibits a unique absorption spectrum with a maximum peak at 865 nm and a shoulder at 700 nm. The isolate was tentatively identified as *Serratia marcescens* Strain Dr.Y6 based on carbon utilization profiles using Biolog GN plates and partial 16s rDNA molecular phylogeny.

Keywords *S. marcescens* · Molybdate-reduction · Molybdenum blue

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Introduction

Bioremediation using microbial enzymes to treat heavy metal pollution is a revolutionary process that has grown extensively in this era of biotechnology. The microorganisms involved in the process come from a variety of genera. Metals that could be detoxified include mercury, chromium, molybdenum, arsenic, lead, copper, uranium, selenium, bismuth, tungsten [1]. Molybdenum reduction by microbe is an interesting phenomenon because the product shows an intense blue allowing the progress of reduction to be observed. Molybdate reduction by microbes have been reported since the last 100 years [2–6]. It was not until 1985 that Campbell et al. [7] revitalizes the phenomenon of molybdate reduction in *Escherichia coli* K12. In 1988, Sugio et al. reported on the reduction of molybdate into molybdenum blue by *Thiobacillus ferreoxidans* Strain AP19–3 [8]. The reducing activity was initially only attributed to the enzyme sulphur/ferric ion oxidoreductase (SFORase). However, further studies showed that aside from enzymatic reduction, Fe^2, which is present in the media, could also chemically reduce molybdate to Mo-blue under acidic conditions [9]. In 1993, Ghani et al. reported that a heterotrophic bacterium, *Enterobacter cloacae* strain 48 (EC 48), could also reduce molybdate to molybdenum blue. The molybdenum blue product adheres strongly to cells indicating that removal of the metal from solution has bioremediation potential [10].The scarcity of the report on molybdate reduction is reflected in the citational absence of the works of Campbell et al. [7] by Sugio et al. [8] and Ghani et al. [10]. For the past 25 years, studies on molybdate reduction to molybdenum blue were carried out In *E. coli* and the local bacterium EC 48 [10–14]. The most recent review on molybdenum reduction to molybdenum blue has also confirmed this [15].

In Malaysia, molybdenum in the form of molybdenite is mined as a by-product of copper mining, and there have been reports of several cases of pollution caused by accidental leakage of pipe-carrying metal system and leaching of the metals from the mining site causing contamination of paddy fields as big as 2,000 acres and the Ranau River [16]. Hence, there is a need to isolate and characterize more local molybdate-reducing bacterium for the purpose of bioremediation. In this work, we report on the characterization and isolation of a molybdate-reducing bacterium, *Serratia marcescens* Strain Dr.Y6 from Malaysian soil.

Materials and Methods

Chemicals

All reagents were of analytical grade. 12-Phosphomolybdate was obtained from Sigma-Aldrich Chemical Co., St. Louis, USA.

Isolation of Molybdate-Reducing Bacterium

Soil samples were taken (5 cm deep from topsoil) near the State Museum in the city of Taiping, State of Perak, Malaysia. The soils were taken from a metal-recycling ground adjacent to the State Museum. Five grams of a well-mixed soil sample were suspended in 45 ml of 0.9% saline solution. A suitable serial dilution aliquot (0.1 ml) of soil suspension was spread plated onto an agar of low phosphate (2.9 mM phosphate) media (pH 7.0) containing glucose (1%), $(NH_4)_2SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.5%), yeast

extract (0.0.5%), $Na_2MoO_4.2H_2O$ (0.242%) and Na_2HPO_4 (0.05%) [10]. Glucose was autoclaved separately. Growth in liquid media uses the same media as in the solid media above. Molybdenum blue is produced in this media but not at high phosphate media (100 mM phosphate). The only difference between the high and low phosphate media is the phosphate concentration. Blue colonies appearing on the low phosphate agar media signify the presence of molybdenum-reducing bacteria. One single colony exhibiting the strongest blue intensity observable by eye was inoculated into 50 ml of low phosphate media and incubated at 30 °C for 24 h. The production of molybdenum 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.

16s rDNA Gene Sequencing and Phylogenetic Analysis

Genomic DNA was extracted from bacterial colonies by alkaline lysis. Polymerase chain reaction (PCR) amplification was performed using a 9700 thermal cycler (PE Biosystems Japan, Tokyo). The PCR mixture contained 0.5 pM of each primer, 200 μM of each deoxynucleotide triphosphate, 1× reaction buffer, and 2.5 U of Taq DNA polymerase (Promega) to achieve a final volume of 50 μ1. 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 [17]. 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 1,279 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 the following accession number DQ226207.

Phylogenetic Analysis

A multiple alignment of 19 16s rRNA gene sequences closely matches Strain Dr.Y6 was retrieved from GenBank and was aligned using ClustalW [18] with the PHYLIP output option. The alignment was checked by eye for any obvious misalignments. Alignment positions with gaps were excluded from the calculations. 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 the outgroup in the cladogram. Evolutionary distance matrices for the neighbor-joining/ UPGMA method were computed using the DNADIST algorithm program. The program reads in nucleotide sequences and writes an output file containing the distance matrix. The model of nucleotide substitution is from Jukes and Cantor [19]. Phylogenetic tree (Fig. 1) was inferred by using the neighbor-joining method of Saitou and Nei [20]. With each algorithm, confidence levels for individual branches within the tree were checked by repeating the PHYLIP analysis with 1,000 bootstraps [21] by the SEQBOOT program in the PHYLIP package. Majority rule (50%) consensus trees were constructed for the

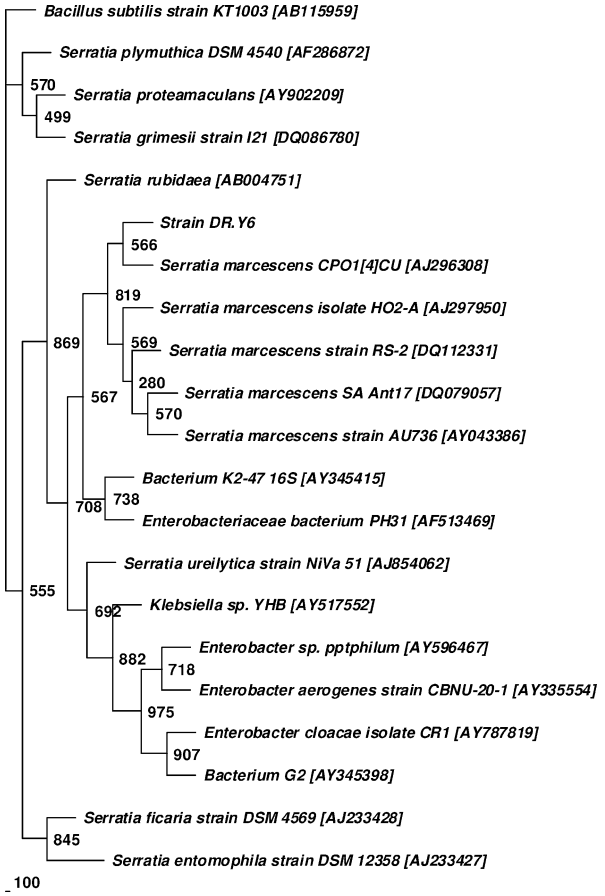

Fig. 1 A phylogram (neighbor-joining method) showing genetic relationship between Strain Dr.Y6 and other related reference microorganisms based on the 16s rRNA gene sequence analysis. Species names are followed by the accession numbers of their 16s rRNA sequences. The *numbers* at branching points or nodes refer to bootstrap values, based on 1,000 re-samplings. *Scale bar* represents 100 nucleotide substitutions. *Bacillus subtilis* strain KT1003 is the outgroup

topologies found using a family of consensus tree methods called the Ml methods [22] using the CONSENSE program, and the tree was viewed using TreeView [23].

Results and Discussions

Identification of the Isolate

A moderate bootstrap value (56.6%) is seen when Strain Dr.Y6 is associated to *S. marcescens* Strain CPO14CU indicating that the phylogenetic relationship between the species is moderately strong (Fig. 1). The identifications performed by Biolog GN give conclusive identification to *S. marcescens* with high probability (99.9%), similarity (0.8), and distance (3.5) values. Thus, this bacterium is assigned tentatively as *S. marcescens* Strain Dr.Y6. In an earlier work, another species, *Serratia* sp., was reported to be able to reduce ammonium molybdate to molybdenum blue [2]. Most of the reported molybdenum-reducing bacteria are heterotrophs with an overwhelming majority belonging to the family of Enterobacteriaceae [2, 4, 7, 10] with the exception of *T. ferreoxidans* [8]. This feature is probably due to the ability of the bacteria belonging to this family to ferment simple sugars resulting in the lowering of pH and inducing the formation of phosphomolybdate.

The Effects of Nitrogen and Carbon Sources on Molybdate Reduction

Since molybdate reduction in the heterotrophic bacterium EC 48 has been reported to be growth-associated [10], factors that increase growth would also increases molybdate reduction. We studied the effect of several nitrogen sources on the cellular growth and molybdate reduction in *S. marcescens* Strain Dr.Y6. Different nitrogen sources such as ammonium sulfate, nitrate, nitrite, alanine, asparagine, aspartic acid, valine, cysteine, glutamic acid, glycine, histidine, leucine, OH-proline, oxaloacetate, alpha-ketoglutarate, and urea were used at an initial concentration of 0.3% (*w*/*v*) in low phosphate media containing 10 mM molybdate to study their effect on the molybdate reduction efficiency of bacterium. All of the nitrogen sources supported cellular growth to a different degree, but only ammonium sulfate, glycine, glutamic acid, and OH-proline (in the order of decreasing efficiency) supported molybdate reduction with ammonium sulfate giving the highest amount of molybdenum blue after 24 h of incubation (Table 1). The effect of different

Table 1 Cellular growth and molybdate reduction using various nitrogen and carbon sources.

Nitrogen and carbon sources	Log CFU/ml	Abs 865 nm
a-ketoglutarate	3.100±0.000	–
Alanine	3.555±0.001	–
Ammonium sulfate	3.533±0.043	1.370±0.270
Asparagine	3.694±0.003	–
Aspartic acid	3.800±0.012	–
Cysteine	3.629±0.025	–
Glutamic acid	3.672±0.047	0.810±0.160
Glycine	3.618±0.095	0.872±0.000
Histidine	3.582±0.016	–
Leucine	3.556±0.020	–
Nitrate	3.229±0.005	–
Nitrite	3.141±0.006	–
OH-proline	3.543±0.042	0.436±0.001
Oxaloacetate	3.146±0.005	–
Urea	3.555±0.024	–
Valine	3.399±0.005	–
Acetate	3.137±0.004	–
Citric acid	3.004±0.008	–
Formate	3.055±0.000	–
Fructose	3.446±0.030	–
Glucose	3.384±0.040	0.749±0.122
Glycerol	3.526±0.025	0.680±0.048
Lactose	3.163±0.002	–
Maltose	3.404±0.013	1.397±0.494
Mannitol	3.497±0.019	–
Starch	3.162±0.028	–
Sucrose	3.352±0.021	2.454±0.426
Tartarate	3.180±0.005	–

Strain DR.Y6 was grown for 24 h in low phosphate liquid medium containing 10 mM molybdate and various nitrogen sources at the final concentration of 0.2% (*w*/*v*). The carbon source is 0.2% (*w*/*v*) glucose. When the carbon sources were varied, ammonium sulfate at 0.3% (*w*/*v*) serves as the nitrogen source. Molybdate reduction is considered negligible if the absorbance at 865 nm is below 0.020. Error bars represent the standard deviations between three determinations

concentrations of ammonium sulfate in the range of 0–1% (*w*/*v*) in low phosphate media on the molybdate reduction efficiency of bacterium was evaluated. In the absence of any ammonium sulfate supplement, no molybdate reduction was observed in the form of molybdenum blue measurable at the wavelength of 865 nm, whereas optimum reduction was achieved at 0.3% (*w*/*v*) after 24 h of static incubation. Incidentally, ammonium sulfate at this concentration was used in the preliminary screening media. Further increase in ammonium sulfate concentration reduces molybdate reduction capability (data not shown). Previously, ammonium sulfate is used as the nitrogen source for molybdenum reduction in EC 48. In *E. coli* K12, tryptone broth was used as the complex nitrogen sources with or without nitrate and nitrite as additional inorganic nitrogen sources. Similar to the findings in *E. coli* K12, both nitrite and nitrate did not support molybdenum reduction in this bacterium.

Different carbon sources such as acetate, formate, glycerol, citric acid, lactose, fructose, glucose, mannitol, tartarate, maltose, sucrose, and starch were used at an initial concentration of 0.2% (*w*/*v*) in low phosphate media containing 10 mM molybdate to study their effect on the molybdate reduction efficiency of bacterium. All of the carbon sources supported cellular growth, but only sucrose, maltose, glucose, and glycerol (in decreasing order) supported molybdate reduction after 24 h of incubation (Table 1). Ghani et al. [10] also reported that sucrose gives the highest rate of molybdate reduction. Campbell et al. [7] reported that the best carbon source that could support molybdate reduction in *E. coli* K12 is glucose, although sucrose was not one of the carbon sources tested. The organic carbon sources are considered essential medium supplements for the regeneration of nicotinamide adenine dinucleotide (reduced form) that acts as electron donor for the reduction of molybdate by microorganisms [10]. The effect of different concentrations of sucrose in the range of 0–10% (*w*/*v*) in low phosphate media on the molybdate reduction efficiency of bacterium was evaluated. Optimum concentration of sucrose for molybdate reduction is 1.0% (*w*/*v*) after 24 h of static incubation (data not shown).

Cellular growth and molybdate reduction using various nitrogen and carbon sources (Table 1). Strain DR.Y6 was grown for 24 h in low phosphate liquid medium containing 10 mM molybdate and various nitrogen sources at the final concentration of 0.2% (*w*/*v*). The carbon source is 0.2% (*w*/*v*) glucose. When the carbon sources were varied, ammonium sulfate at 0.3% (*w*/*v*) serves as the nitrogen source. Molybdate reduction is considered negligible if the absorbance at 865 nm is below 0.020. Error bars represent the standard deviations between three determinations.

The Effects of Molybdate and Phosphate Concentrations on Molybdate Reduction

The effect of phosphate and molybdate concentrations on molybdate reduction has been found to be instrumental in molybdate reduction [7, 10]. Thus, this study is very important to ascertain the limits of phosphate ion as a molybdate reduction inhibitor in *S. marcescens* Strain Dr.Y6. Phosphate on molybdate reduction was studied using phosphate concentrations from 2.5 to 100 mM and fixing molybdate concentration at 10 mM. The effect of molybdate concentrations on molybdate reduction was studied using molybdate (sodium molybdate) concentrations ranging from 5 to 30 mM while fixing phosphate concentration at 5 mM.

The optimum phosphate concentration for molybdate reduction in *S. marcescens* Strain Dr.Y6 is 5 mM. Reduction decreases rapidly at much high phosphate concentration and is totally inhibited at 100 mM phosphate (Fig. 2). The effect of molybdate concentrations on

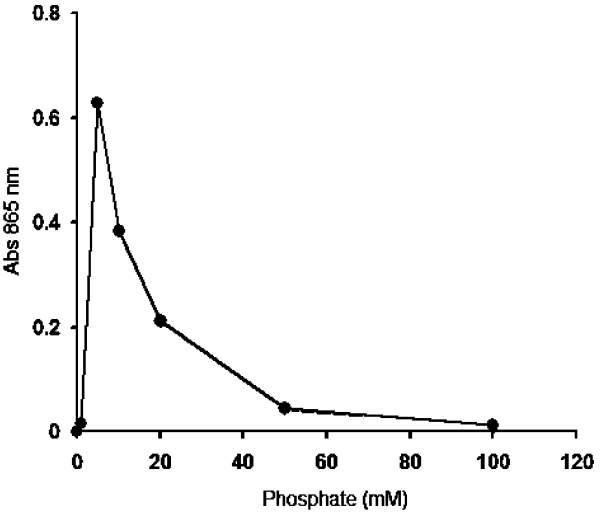

Fig. 2 The effect of phosphate concentrations on molybdate reduction by Strain Dr.Y6. *Error bars* represent the standard error of the mean between three determinations

molybdate reduction shows that the molybdate reduction increases linearly as molybdate concentration was increased from 0 to 15 mM and reaches optimum at abroad molybdate concentrations ranging from 15 to 25 mM and is inhibited at 30 mM molybdate (Fig. 3). Campbell et al. [7] reported that the ratio of molybdate to phosphate is much more important than the actual concentrations of molybdate or phosphate per se. Ghani et al. [10] reported that phosphate concentration greater than 0.5 mM inhibits molybdate reduction. It has been suggested that the mechanism of inhibition of molybdate reduction by high phosphate is by maintaining the pH at neutral. At this pH, the formation and stability of phosphomolybdate is severely affected [24].

The Effect of Temperature on Molybdate Reduction

For all organisms, growth is affected by temperature according to a Gaussian distribution. All has a minimum temperature below which growth no longer occurs, an optimum

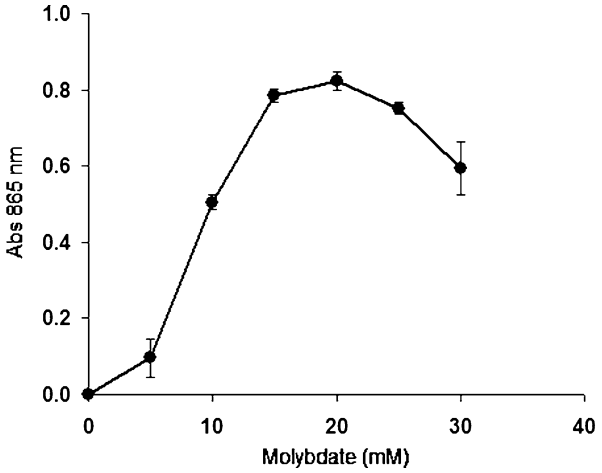

Fig. 3 The effect of molybdate concentrations on molybdate reduction. Strain Dr.Y6 was grown statically for 24 h in low phosphate liquid medium containing media (pH 7.0) containing sucrose (1%), $(NH_4)_2SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.5%), yeast extract (0.05%), $Na_2MoO_4.2H_2O$ (0.242%), and Na_2HPO_4 (0.05%). *Error bars* represent the standard error of the mean between three determinations

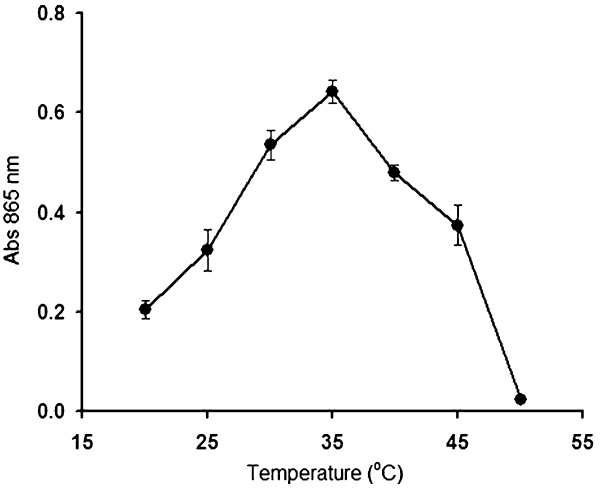

Fig. 4 The effect of temperature on molybdate reduction. Strain Dr.Y10 was grown statically for 24 h in low phosphate liquid medium (pH 7.0) containing sucrose (1%), $(NH_4)_2SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.5%), yeast extract (0.05%), $Na_2MoO_4.2H_2O$ (20 mM), and Na_2HPO_4 (5 mM). *Error bars* represent the standard error of the mean between three determinations

temperature at which growth is most rapid, and a maximum temperature above which growth is not possible. The study of temperature optima for the growth of microbes would be very useful for bioremediation purposes. Although generally it is not possible to change temperature when conducting bioremediation works on the field, screening for indigenous microbes for local bioremediation works is the norm because these microbes would have an optimum temperature close to the temperature of the site chosen for bioremediation. The effect of temperature on the molybdate reduction efficiency of bacterium was studied at temperature ranging from 20 to 50 °C. Molybdate reduction is optimum at 35 °C and drops rapidly after this and is completely inhibited at 50 °C (Fig. 4). Ghani et al. [10] employed 30 °C for growing cells of EC 48, while Campbell et al. [7] reported that the optimum temperature range for molybdate reduction in *E. coli* strain K12 is from 30 to 36 °C. The high optimum temperature for activity suited the Malaysian equatorial climate.

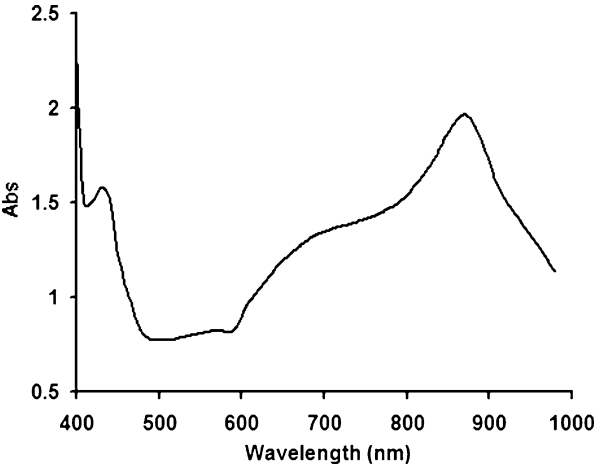

Fig. 5 Scanning spectrum of molybdenum blue from Strain Dr.Y6

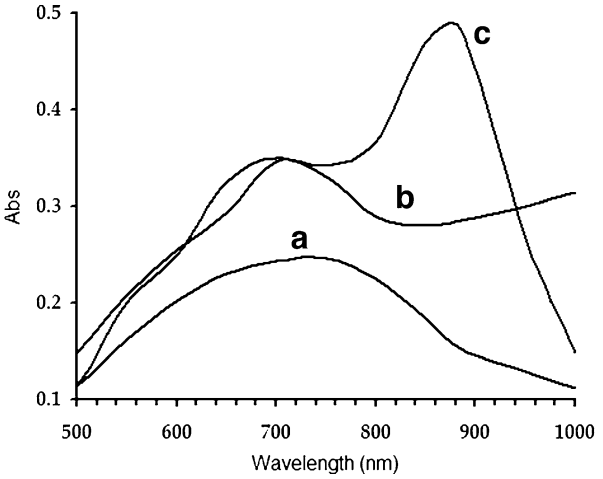

Fig. 6 Visible absorption spectra of molybdenum blue from molybdosilicate (**a**) modified and adapted from Glenn and Crane [24], molybdosulfate (**b**), taken from Hori et al. [28], and phosphomolybdate (**c**) taken from Yoshimura et al. [29]

Molybdenum Blue Spectrum, Its Production and pH Profile During Cellular Molybdate Reduction

The molybdenum blue produced by the bacterium shows the same characteristics absorption profile to that of EC 48 [12], with a maximum peak at 865 nm and a shoulder at 700 nm. The overall visible spectrum for molybdenum blue from this bacterium increases as the amount of molybdenum blue increases. The characteristics absorption profile of Strain Dr.Y6 (Fig. 5) is similar to the molybdenum blue produced by the phosphate determination method and is not similar to other heteropolymolybdate species such as sulfomolybdate and silicomolybdate (Fig. 6). The characteristics spectrum with an absorption optimum in between 860 and 880 nm and a shoulder at approximately 700 nm is very specific to reduced phosphomolybdate and is an indication of the presence of phosphomolybdate [25]. This is why we proposed earlier that the reduction of molybdate to molybdenum blue in EC 48 proceeds first by the formation of phosphomolybdate before

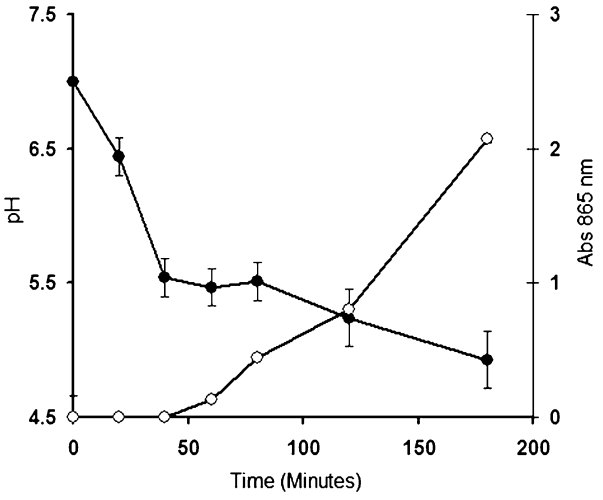

Fig. 7 Changes in pH (*filled circle*) of the media during the course of molybdenum blue production (*open circle*). *Error bars* represent the standard error of the mean between three determinations

the reduction to molybdenum blue by enzyme(s) takes place. Using phosphomolybdate as the molybdenum-reducing enzyme substrate instead of the original molybdate resulted in shortening of the molybdenum-reducing enzyme assay from several hours to a few minutes. The resultant reduced phosphomolybdate from enzymatic reduction also has a similar absorption spectrum to cellular-reduced molybdate [14].

The pH profile during molybdate reduction to molybdenum blue (Fig. 7) shows that molybdate reduction is preceded by a reduction in pH before reduction takes place. This was also observed in EC 48 [12]. The lowering in pH is an important event for molybdate conversion to phosphomolybdate before the latter's reduction [26, 27]. Initially, it was proposed that molybdate (Mo^{6+}) was first reduced to Mo^{5+} by molybdenum reductase (Mo-reducing enzyme) before the joining of phosphate anions forming Mo-blue in EC 48 [8, 10]. However, the chemistry of molybdate dictates that under acidic conditions and in the presence of phosphate anions, phosphomolybdate would form [26, 27]. Acidic conditions are provided by static growth on glucose or other fermentable carbon sources. As the finding in this work is similar to that from EC 48, this evidence suggests that molybdate reduction in this bacterium is likely to proceed via a phosphomolybdate intermediate.

Conclusions

In this work, we described the isolation and characterization of another molybdenum-reducing bacterium. We have studied the effect of various parameters such as nitrogen and carbon sources, temperature, molybdate, and phosphate on molybdate reduction in *S. marcescens* Strain Dr.Y6. This knowledge is not only important for contributing to the fundamental understanding of the mechanism of reduction but will also be beneficial in the area of bioremediation of molybdenum. We found that the optimum conditions are quite similar to previously published works from *E. coli* K12 and EC 48. We also observed a similar spectrum of the molybdenum blue produced by *S. marcescens* Strain Dr.Y6 to that of EC 48. This and the observation that the pH is decreased before molybdenum blue is produced led us to the conclusion that phosphomolybdate has an important intermediary role during molybdate reduction to molybdenum blue. Currently, work is underway to purify the molybdenum-reducing enzyme from *S. marcescens* Strain Dr. Y6.

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