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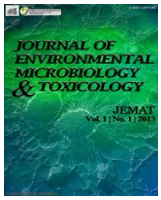

Short communication

Isolation of Mo-reducing Bacteria in Soils from Pakistan

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Abstract

Molybdenum is a heavy metal that is very toxic to ruminants and has been shown to inhibit spermatogenesis in catfish and mice at levels as low as several parts per million. In this work we report the first isolation of molybdenum-reducing bacteria from Pakistani soils. Out of the ten isolates, three were able to reduce molybdenum to molybdenum blue at molybdenum concentration in excess of 50 mM. Several strains were able to reduce molybdenum in the presence of the toxic heavy metals copper and mercury. All isolates were resistant to chromium concentration as high as 10 mM. The characteristics of these strains make them very useful in bioremediation works in Pakistani soils.

INTRODUCTION

Molybdenum is a heavy metal with low toxicity to humans but is very toxic to ruminants with levels as low as several parts per million causing scouring and in certain cases deaths [1]. Recently works have also shown that molybdenum inhibits spermatogenesis in catfish and mice at levels as low as several parts per million [2–5]. Molybdenum pollution has been recorded at an ever increasing level globally. Hence the removal of molybdenum from soils and aquatic bodies is urgently needed. In the past decades researchers have focused on bioremediation as an environmental friendly and low cost method to solve this problem. Bioremediation is one of the ways to remove toxic metals from the environment [6].

Currently, most of the Mo-reducing bacteria are isolated from Malaysian soils [7,7–16] with the exception of *E. coli* K12 [17]. There is no report on the isolation of Mo-reducing bacterium from anywhere else. Hence we report the first isolation of Mo-reducing bacteria from Pakistani soils. This is a preliminary report on the isolation of such a bacterium before more detailed work is done in the future.

MATERIALS AND METHODS

Isolation of molybdenum-reducing bacterium

Samples were collected from the Hazara district, Pakistan in 2013. Soils were collected 15-20 centimetres (cm) beneath the surface and were placed in sterile screw-capped vials. The samples were immediately placed in a freezer and stored at -20°C until returned to the laboratory for further examination. About five grams of soil sample that was well-mixed were suspended in 45 mL of 0.9% saline solution. Suitable serial dilutions of soil suspension were then spread plated onto an agar of low phosphate (5 mM phosphate) medium (LPM) (2.8 mM phosphate) (pH 7.0). The medium (w/v) consisted of glucose (1%), $MgSO_4.7H_2O$ (0.05%), $(NH_4)_2SO_4$ (0.3%), NaCl (0.5%), $Na_2MoO_4.2H_2O$ (0.242%), yeast extract (0.05%) and $Na_2HPO_4.2H_2O$ (0.089%) to isolate molybdenum-reducing bacteria [13]. After 24 hours of incubation at room temperature several white and blue colonies appeared. Ten colonies exhibiting the strongest blue intensity were then inoculated into 50 mL of low phosphate medium and incubated at room temperature for 24 hours statically. Incubation under aerobic conditions gave lower amount of Mo-blue compared to static incubation.

The effects metal ions

Metal ions such as Cr^{6+} ($K_2Cr_2O_7$, BDH), Ag^+ ($AgNO_3$, JT Baker), Cu^{2+}($CuSO_4.5H_2O$, JT Baker), Hg^{2+} ($HgCl_2$, JT Baker), Cd^{2+} ($CdCl_2$, JT Baker) and Pb^{2+} ($PbCl_2$, JT Baker) were dissolved in 20 mM Tris.Cl buffer pH 7.0 and were added into culture to the final concentrations of between 1 and 10 mg/L.

Statistical analysis

Values are means ± SE. All data were analyzed using Graphpad Prism version 3.0 and Graphpad InStat version 3.05 available from www.graphpad.com. 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.

RESULT

Production of Mo-blue after 24 hours of static incubation

Ten Mo-reducing bacteria were successfully isolated from soils. Isolates A8 and A9 showed the strongest molybdenum reduction amongst the ten soil isolates whilst isolates A1 and A2 were the weakest reducer. During screening it was observed that about 30-40% of the colonies were not capable to reduce molybdenum to Mo-blue.

Table 1. Isolates were grown at room temperature for 24 hours in 10 ml of low phosphate (5 mM phosphate) liquid medium (pH 7.0) containing (w/v) glucose (1%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.5%), yeast extract (0.05%), $Na_2HPO_4.2H_2O$ (0.089%), ammonium sulphate (0.2%) and 10 mM sodium molybdate. Strength of reduction is semi-quantitatively graded according to the following scores: –, none; X, weak; XX, moderate; XXX, strong.

Isolates	Mo-blue production
A1	x
A2	xx
A3	xx
A4	xx
A5	x
A6	xx
A7	xx
A8	xxx
A9	xxx
A10	xx

Production of Mo-blue at 50 mM molybdenum

In this screening work a high concentration of molybdenum was chosen as a lot of Mo-reducing bacteria isolated so far are tolerant to molybdenum at this concentration but poorly reduce Mo-blue. The results show that isolate A7 gave the strongest molybdenum reduction whilst isolates A1, A2 and A4 were the weakest molybdenum reducer at 50 mM molybdate.

Table 2. Production of Mo-blue in 10 ml of low phosphate media supplemented with 50 mM sodium molybdate by soil isolates. Isolates were grown at room temperature for 24 hours in low phosphate (5 mM phosphate) liquid medium (pH 7.0) containing (w/v) glucose (1%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.5%), yeast extract (0.05%), $Na_2HPO_4.2H_2O$ (0.089%) and ammonium sulphate (0.2%). Strength of reduction is semi-quantitatively graded according to the following scores: –, none; X, weak; XX, moderate; XXX, strong.

Isolates	**Relative reduction**
A1	x
A2	x
A3	xx
A4	x
A5	xx
A6	xx
A7	xxx
A8	xx
A9	xx
A10	xx

The effect of six heavy metals on the reduction of molybdenum was evaluated at two different levels. At 10 mg/L of the heavy metals copper, mercury and cadmium, none of the bacterium could reduce molybdenum whilst only isolate A3 was resistant to silver and only isolate A9 was resistant to lead. All isolates were resistant to chromium with isolate A1 was the most resistant. At 1 mg/L heavy metals, all isolates appear to be not inhibited by chromium. In addition all isolates were resistant to silver and cadmium at markedly reduce potential for molybdenum reduction compared to chromium whilst only three isolates were resistant to each of the heavy metals copper (A1, A3 and A7) and mercury (A1, A6 and A10).

Table 3. Effect of metal ions at 10 mg/L (a) and 1 mg/L (b) on molybdate reduction by isolates. Isolates were grown at room temperature for 24 hours in low phosphate (5 mM phosphate) liquid medium (pH 7.0) containing (w/v) glucose (1%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.5%), yeast extract (0.05%), $Na_2HPO_4.2H_2O$ (0.089%) ammonium sulphate (0.2%) and 10 mM sodium molybdate. Strength of reduction is semi-quantitatively graded according to the following scores: –, none; X, weak; XX, moderate; XXX, strong.

a)

Isolates	Cr	Ag	Cu	Hg	Cd	Pb
A1	xxxx	-	-	-	-	-
A2	xx	-	-	-	-	-
A3	x	x	-	-	-	-
A4	x	-	-	-	-	-
A5	x	-	-	-	-	-
A6	x	-	-	-	-	-
A7	x	-	-	-	-	-
A8	x	-	-	-	-	-
A9	x	-	-	-	-	X
A10	x	-	-	-	-	-

b)

Isolates	Cr	Ag	Cu	Hg	Cd	Pb
A1	xxxx	xx	X	x	x	Xx
A2	xxxx	xx	-	-	x	X
A3	xxxx	x	Xx	-	x	Xx
A4	xxxx	xx	-	-	x	X
A5	xxxx	x	-	-	xx	x
A6	xxxx	x	-	x	x	x
A7	xxxx	xx	X	-	xx	-
A8	xxxx	x	-	-	x	-
A9	xxxx	x	-	-	x	x
A10	xxxx	x	-	x	x	-

DISCUSSIONS

Mo-reducing bacteria reduce sodium molybdate into a colloidal compound called molybdenum blue [18,19] that precipitates together with the cells [20]. The Mo-blue compound has an absorption maximum at 865 nm and a shoulder at 710 nm [13]. Mo-reducing bacterium from Pakistani soils shows variable Mo-reduction capability all was able to reduce molybdenum as high as 50 mM but with varying efficiency. It is important no note that the reduction was carried out at room temperature for all isolates and further optimization for each isolates would be carried out later. The susceptibility of Mo-reducing bacteria to heavy metals has well been documented [21]. The aim of this work is to isolate better resistant Mo-reducers as polluted sites could have several heavy metals present as well as other toxic xenobiotics. Potential isolates with tolerant to the six heavy metals tested at 1 mg/L were isolated I this work and this tolerance is very important as previous isolates showed strong inhibition to many of the six

heavy metals tested especially copper and mercury (Table 1). The characteristics of other Mo-reducing bacterial isolates showed optimal reduction between 20 and 50 mM molybdate (Table 1) and that is the reason as to why 50 mM molybdate was chosen as the initial screening concentration so that newer isolates with higher tolerable molybdate concentration and reduction could be discovered. This is because molybdenum pollution as high as 20 mM have been reported from a molybdenum mining effluents [8] and the presence of other inhibitory heavy metals aside from molybdenum can not be excluded.

Table 1. Previously reported Mo-reducing bacteria and their characteristics.

Bacteria	Optimal Molybdate (mM)	Heavy Metals that inhibit reduction	Author
Bacillus pumilus strain lbna	40	As^{3+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	(7)
Bacillus sp. strain A.rzi	50	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, $Hg^{2+,}$ Co^{2+},Zn^{2+}	(10)
Serratia sp. strain Dr.Y8	50	Cr, Cu, Ag, Hg	(15)
S. marcescens strain Dr.Y9	20	Cr^{6+}, Cu^{2+}, Ag^{+}, Hg^{2+}	(23)
Serratia sp. strain Dr.Y5	30	n.a.	(21)
Pseudomonas sp. strain DRY2	15-20	Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	(11)
Pseudomonas sp. strain DRY1	30-50	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, Hg^{2+}	(8)
Enterobacter sp. strain Dr.Y13	25-50	Cr^{6+}, Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}	(14)
Acinetobacter calcoaceticus strain Dr.Y12	20	Cd^{2+}, Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	(16)
Serratia marcescens strain DRY6	15-25	Cr^{6+}, Cu^{2+}, Hg^{2+}*	(23)
Enterobacter cloacae strain 48	20	Cr^{6+}, Cu^{2+}	(24)
Escherichia coli K12	80	Cr^{6+}	(17)
Klebsiella oxytoca strain hkeem	80	Cu^{2+}, Ag^{+}, Hg^{2+}	(9)

CONCLUSION

In conclusion, we have isolated and characterized several molybdenum-reducing bacteria from Pakistano soil. To the best of our knowledge, this is the first report of Mo-reducing bacterium from Pakistani soils. Ten isolates were characterized for their strength of reduction in low phosphate media, the ability to reduce molybdate at the high concentration of 50 mM and the resistibility of these isolates to toxic heavy metals. Several isolates were able to reduce molybdate at 50 mM molybdate while other isolates showed better resistance to toxic heavy metals such as copper and mercury. The information obtained in this work is not only important for contributing to the understanding of fundamental mechanism of molybdate reduction but will be extremely important in future works on the bioremediation of molybdenum in polar environments.

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