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Original Article

Chromium bioremediation potential of indigenous *Bacillus pumillus* isolated from river water of Bangladesh

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Bangladesh has achieved rapid industrialization in recent years. However, many of these industries lack proper effluent treatment plant and discharge untreated effluent laden with different heavy metals into the major rives that surround these industries, affecting the environment as well as human and animal health. Aiming to develop a sustainable effluent treatment plant, a heavy metal tolerant *Bacillus pumillus* isolated from polluted river water of Bangladesh was studied for its chromium bioremediation potential. Reduction of hexavalent chromium using the S-diphenylcarbazide (DPC) method showed that whole cells of the *Bacillus pumillus* reduced 89.5%, 75%, 73% and 45% of 1.0, 2.5, 5 and 10mg/L Cr(VI) to Cr(III), respectively. This bacterium reduced 100% of 20mg/L Cr(VI) to Cr(III) within 8 hours, in a growth associated pattern. A 20kb plasmid was detected in this *Bacillus pumillus*, and loss of this plasmid did not cause complete impairment of chromium tolerance capacity, though the tolerance efficiency was reduced. The *Bacillus pumillus* studied in the current study therefore shows its potential to develop a sustainable chromium bioremediation method.

Keywords: Chromium, Bioremediation, *Bacillus pumillus*

Introduction

The heavy metal Chromium is used in different industrial purposes in Bangladesh including leather tanning, wood processing, metal polishing etc[1]. Industrial processes like welding on stainless steel, melting at high temperature converts the trivalent chromium to its hexavalent state, while in case of leather tanning, chromium discharged in the environment is oxidized into hexavalent form[2]. Therefore, as most of these industries are situated near the river banks and many of them do not have proper effluent treatment plant, discharge of untreated waste results in high concentration of hexavalent chromium in the river water[1,2,3]. This not only pollutes the river water but also contaminates the aquatic life, agriculture fields, finally allowing chromium to enter human body through the food chain[2].

Among the two different forms of chromium found in nature, the hexavalent form Cr(VI) is toxic, mutagenic and carcinogenic and therefore considered as a pollutant by the US Environmental Protection Agency[4]. Exposure to high concentration of Cr(VI) can cause potentially permanent learning and behavior disorders in children[5]. Consumption or exposure to chromium polluted water can cause skin, gastrointestinal, respiratory, reproductive and genetic disorders in human leading to cancer as well as death[5]. Accumulation of chromium in plants and animals also induce DNA damage and intense oxidative stress resulting in genotoxic and mutagenic effects[5.6]. Therefore, removal of chromium from water is of utmost importance given the impact on public as well as environmental health.

In recent years, transformation of toxic Cr(VI) into harmless Cr(III) by bacterial has gained attention worldwide as an environmental friendly and cost effective approach of chromium removal[7,8,9,10]. Transformation of Cr(VI) to Cr(III) allows bacteria to tolerate high concentration of chromium and has been reported by many bacterial species including *Bacillus pumillus , Pseudomonas, Proteus*, *Acinetobacter* spp., *Burkholderia*. The genetic basis to chromium tolerance in bacteria are chromosomal, plasmid mediated or both[11,12,13].

In view of the above, the current study investigated the potential of a Chromium resistant *Bacillus pumillus*, isolated from the polluted river water of Bangladesh[14] to transform Cr(VI) to Cr(III) with aim to develop a sustainable chromium bioremediation method.

Material and method

Chromium reduction by whole cells in buffer

The hexavalent chromium reduction assay was performed following method described by Ilias *et al.* [15](2011). Six aliquots of bacterial cells (1.5 ml) were harvested from overnight culture of *Bacillus pumillus* grown in 50 ml Lauria Bertani (LB) medium supplemented with 50ppm chromium (as $K_2Cr_2O_7$) with 150 rpm agitation at 37°C. The cell pellets from each aliquote was washed twice with phosphate buffer (100 mM, pH 7.0) and were resuspended in 0.5 ml phosphate buffer. To initiate Cr(VI) reduction, 0.5 ml of $K_2Cr_2O_7$ of different concentrations (1mg/L, 2.5 mg/l; 5mg/l; 10 mg/l and 20mg/L) in phosphate buffer

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was added into five different cell suspensions and one cell suspension was used as control with no addition of chromium. Followed by incubation at 37°C for 45 min, supernatant from each cell suspenstion was collected and analysed for residual Cr (VI) determination colorimetrically following the S-diphenylcarbazide (DPC) method[15]. Independent experiment was conducted in triplicates.

Time course assay of Cr(VI) reduction

The time course of growth and Cr(VI) reduction/transformation for the isolate was analysed as described by Ilias *et al.* (2011)[15]. The *Bacillus pumillus* was grown in 100 ml LB broth supplemented with 20 mg/l $K_2Cr_2O_7$ and was incubated at 37°C and 150 rpm. Samples (1.5 ml) were withdrawn at 2h intervals and were centrifuged (Eppendorf, Germany) at 10,000 rpm for 5 min. The supernatant was assayed for residual Cr(VI) concentration colorimetrically following the S-diphenylcarbazide (DPC) method. The growth of the bacteria was monitored measuring OD at 600 nm.

Reduction of Cr(VI) in cell fractions

This experiment was conducted following the method described by Ilias *et al*., (2011) with slight modification. Cell pellets and culture supernatant were collected from the *Bacillus pumillus* grown overnight in 100 ml of LB broth with 100 ppm of Cr (as $K_2Cr_2O_7$) at 37^0C with orbital shaking (150 rpm). The cell pellets was resuspended in 30 ml phosphate buffer (100 mM, pH 7.0) and was disrupted using sonication. Cell extract supernatant was collected by centrifugation at 14,000 rpm at 4°C for 20 min following sonication. Cell lysate was also resuspended in 30 ml phosphate buffer. To determine the chromium reduction activity of these cell fractions, 0.5 ml of culture supernatant, cell extract supernatant or cell lysate was added to a reaction mixture contained 2 mg/l Cr(VI) as $K_2Cr_2O_7$ in 0.5 ml of 100 mM phosphate buffer, pH 7.0. After incubation for 1 hour at 37°C in water bath, residual Cr(VI) concentration was measured for each reaction mix following the DPC method, as previously described.

Plasmid isolation

For plasmid isolation method described by Brinboin and Dolly (1979) was followed with slight modification[16]. Overnight culture of the bacteria was harvested at 10000 rpm and the pellets were thoroughly suspended in 100 µl solution I (50 mM glucose , 25 mM Tris-HCl and 10 mM EDTA pH 8.0) following incubation at room temperature for 10 min. Then 100 µl of lysis solution (0.2N NaOH, 1% SDS) was added and mixed gently. The tubes were kept on ice for 5 minutes and then 150 µl of ice cold solution III (3M Potassium acetate, 5 M glacial acetic acid pH 4.8, TE buffer 10mM Tris-HCl and 1mM EDTA pH 8.0) was added and kept on ice for 5 minutes. The clear supernatant was taken into fresh Eppendorf tubes and mixed with twice the volume of cold 95% ethanol followed by incubation for 2 minutes at room temperature. The precipitated plasmid DNA was collected by centrifugation for 10minutes at 12000 rpm. The dried plasmid DNA was dissolved in 50 µl Tris-EDTA (TE) buffer.

Plasmid curing

This experiment was performed according to the method described by Samanta *et al.,* (2017)[17]. An overnight culture of the bacteria grown in LB broth containing 100 ppm Chromium was diluted to 10^3 cells/ml with LB broth containing 40mg Ethidium Bromide and was grown for 72 hours at 37°C 150 rpm . The treated cells were plated on LB agar and incubated at 37°C for 24 hours. Plasmid cured colonies were selected by inoculating colonies on LB agar with and without chromium. Cured cells were recovered and plasmid profile was analyzed by agarose gel electrophoresis. The treated cells were diluted with normal saline and appropriate dilution (0.1 ml) were plated on LB agar (with and without chromium) and incubated at 37°C for 24 hours. Colonies that grew on LB agar, but failed to grow on LB agar supplemented with 300ppm Cr were selected as cured cells. These cells were recovered and subjected to further investigation.

Results

Hexavalent chromium reduction by Bacillus pumillus

Chromate reduction activities of *B. pumillus* using different chromium concentrations revealed that at 1 mg/L Cr(VI) concentration (as $K_2Cr_2O_7$), 89.5% hexavalent chromium was transformed (Figure 1). With increase in chromium concentration to 2.5 mg/L, 5 mg/L and 10 mg/L, reduction of Cr(VI) was transformed to 75%, 73% and 45% respectively (Figure 1). No hexavalent chromium reduction was observed when concentration was 20mg/L chromium.

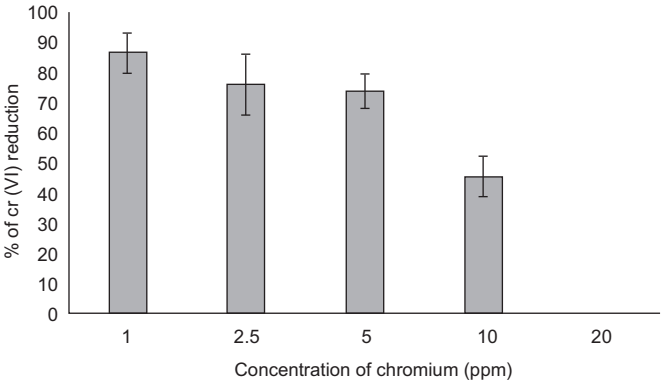

Fig. 1. *Reduction of hexavalent Chromium by the Bacillus pumillus*

Time course of Cr(VI) detoxification

Time course assay of chromium reduction showed that as the *B. pumillus* continued to grow, residual Cr(VI) decreased with time (Figure 2). Within 12 hours, 100% of the Cr(VI) present in the medium was reduced by *B. pumillus*, which suggests that chromium reduction was growth associated.

Reduction of hexavalent chromium by different cell fractions

The cell extract supernatant and culture supernatant of the *B. pumillus* reduced 18.76% and 44.64% Cr(VI), respectively. No reduction was observed when cell lysate was used. It could be presumed that the chromium reduction activity is extracellular.

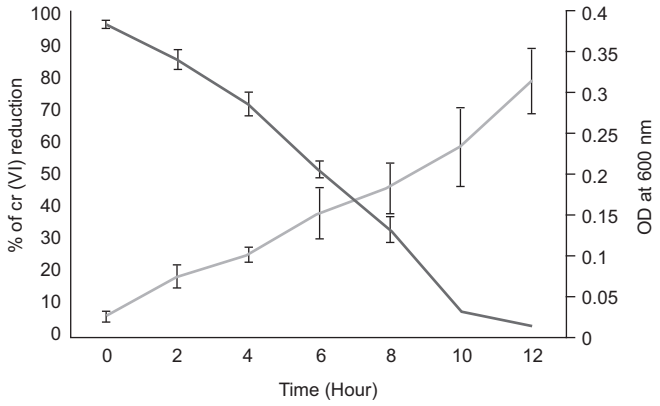

Fig. 2. *Time course assay of Cr(VI) reduction. The blue line shows reduction of Cr(VI) and red line shows growth of the bacteria*

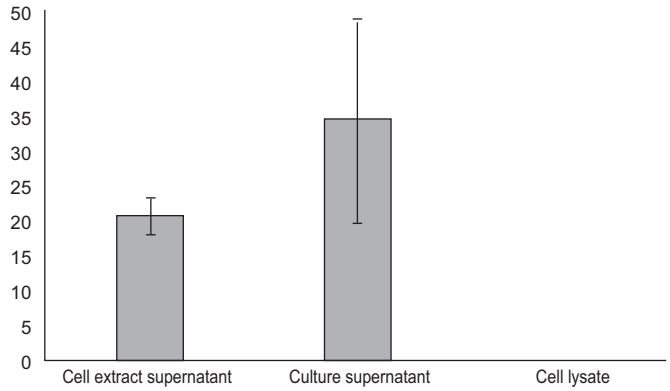

Fig. 3. *Reduction of Cr(VI) by cell fractions*

Plasmid and chromium tolerance

A plasmid of approximately 20kb in size was detected in the *B. pumillus* (Figure 4a). When this plasmid was cured using ethidium bromide, the cured cells were not as efficient as the wild type cells to tolerate high chromium concentration (Figure 4b).

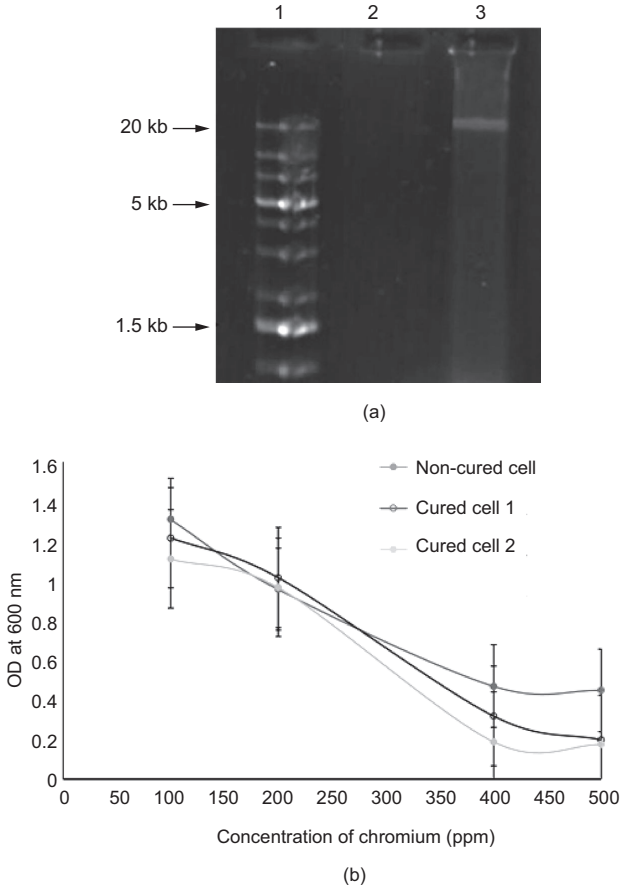

Fig. 1. *Association of plasmid with chromium tolerance; (a) Agarose gel electrophoresis of plasmid DNA. From left: Lane 1- 1kb DNA ladder, Lane 2-negative control, Lane 3- B. pumillus. (b) Tolerance of wild-type and cured cells of B. pumillus to increased concentration of chromium. Green, blue and yellow lines indicate non-cured wild type, and growth from two different colonies from cured cells, respectively.*

Discussion

This study analyzed the chromium bioremediation potential of an indigenous *B. pumillus* isolate aiming to develop a chromium bioremediation method to remove this toxic heavy metals from polluted water of Bangladesh. The bacterium could transform different concentrations of hexavalent chromium (up to10mg/ml) within 45 minutes at varying efficiencies, but failed to reduce 20mg/ml chromium within this time period. However, complete reduction of 20mg/ml chromium was achieved within 12 hours in a growth associated manner. This data reflects the observation by Mishra *et al.,* (2012) who reported that *an increase in incubation time from 30 minutes to* 8 hour resulted in increase in Cr(VI) reduction from 30% to 100% by chromium tolerant *Lactobacillus* sp.[19]. Increase in incubation time also increased Cr(VI) reduction by *P. entomophila* MAI4 significantly [20]. It shows that the concentration of hexavalent chromium and amount of cells present in the experiment condition influence reduction of hexavalent chromium by the *B. pumillus.* This concentration dependent Cr(VI) reduction is possibly due to the mutagenic and toxic effects of high concentration of Cr(Vi) on bacterial cell. Bacterial cells might initially need to adjust to the chromium toxicity before initiating its reduction reaction until the growth was increased to reduce chromium with more efficiency. Similar observation was reported by Rafiqullqh *et al.,* who showed that chromium tolerant *Staphylococcus aureus* and *Pediococcus pentosaceus,* reduced 100% hexavalent chromium within 24 hours[21].

Several researchers reported a direct association between plasmid and its tolerance to different heavy metals including chromium[17,22,23]. In many studies, loss of plasmid completely disrupted tolerance to heavy metals[17]. The *B. pumillus*. investigated in the current study also harbors a plasmid, however unlike other studies, curing of this plasmid did not cause complete inhibition of chromium tolerance. Rather chromium tolerance efficiency of the plasmid cured cells was decreased as compared to the wild-type cells. This suggests that in this *B. pumillus*, genes associated with chromium tolerance are not solely present on

plasmid, rather they are distributed on both chromosomes and plasmids. Similar distribution of heavy metal tolerance genes has been observed in *R. sphaeroides*, which had its 375 heavy metal resistance genes distributed on the chromosome as well as plasmids[24].

The current study analyses the potential of an indigenous, novel chromium tolerant bacteria for chromium bioremediation application. Detailed study on this isolate might enable developing an economic and efficient effluent treatment plant to remove toxic Chromium from tannery effluents, hence preventing water pollution and ensuring supply of safe water in Bangladesh.

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