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Identification of Bacteria Capable of Chromium Bioremediation from the Environment

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Abstract

With the increased urbanization and industrial development, the environment is getting polluted severely day by day. In Bangladesh, the capital city Dhaka is surrounded by a circular river system, most of which are highly polluted due to discharge of municipal and industrial untreated waste water that has been overloaded with different types of toxic heavy metals. Cr (VI) being used in various activities of industries and its improper treatment lead to contamination of the environment. This study aimed to isolate bacteria with chromium bioremediation potential from the environment. A total of 71 bacteria were isolated, of which all grew in the presence of 100 ppm, 93% in 200 ppm, 46% in 400 ppm, 6% in 600 and 4% in 1000 ppm chromium. Fourteen bacteria selected for chromium reduction by Diphenyl Carbazide Assay showed 23-89% reduction. Of these 14 isolates, 5 were tested for biosorption of chromium. Variable sorption of upto 69% was observed. The 16s rDNA of 6 candidate bacteria were sequenced. These isolates were identified as *Kurthia gibsonii, Acinetobacter schindleri, Pseudomonas aeruginosa, Exigubacterium* sp., *Rhodococcus* sp., and *Bacillus* sp. The reduction potential was found to be highest for *Bacillus* species (78%) and lowest for *Kurthia gibsonii* (4.8%). This study has successfully identified six potential bacterial candidates for the bioremediation of chromium (VI). Further research into the mechanisms adopted by these bacteria and genetic manipulation will help to formulate a single or mixture of isolates that can efficiently remove hexavalent chromium from polluted water.

Introduction

Wastewater from industrial applications contain potentially toxic metals and anonymous organics that cause alarming environmental pollution worldwide (Saha and Ali 2001). Different tannery industries of Bangladesh frequently use chromium in spite of the fact that it is toxic. Discharge of chromium in waste water without proper treatment leads to

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contamination of water bodies, endangerment of aquatic lives, and health hazard (Jobby et al. 2018). It is a public health concern that needs to be addressed to reduce toxicity or chromium load from industrial effluents (Pavithra et al. 2019). There are about 250 leather tanning industries at Hazaribagh and Hemayetpur in Dhaka city, the capital of Bangladesh, which discharge liquid and solid wastes into canals and rivers, thus increasing the amount of Cr in the surrounding water bodies (Ilias et al. 2011). Chromium salts containing Cr (III) is used primarily by tannery industries as tanning agents during leather processing. Under suitable conditions, it can be readily oxidized to Cr (VI), a more toxic form of chromium (Lofrano et al. 2013). Human Rights Watch reported that the tannery wastewater from 47 tanneries in Hazaribagh contained extremely elevated levels of total chromium (4043 mg/l). The tannery effluents in Chittagong, the second biggest city in Bangladesh, contained hexavalent chromium at 70.33 mg/l (Islam et al. 2011). Cr (VI) was also found in deep tube wells in the Hazaribagh area, which exceeded the Bangladesh standard limit (0.05 mg/l) of chromium in drinking water (Saha and Ali 2001). As Cr (VI) is toxic and highly soluble in water, it can pass rapidly through cell membranes and eventually interact with proteins and nucleic acids. Thus, the accumulation of toxic heavy metals in humans leads to carcinogenesis, mental retardation, renal malfunction, and other abnormalities (Markiewicz et al. 2015). The prevalence of diseases such as scabies (73.9%), gastrointestinal problem (71.7%), diarrhoea (71.7%), asthma (49.9%), eye problems (46.7%), and high blood pressure (52.2%) were reported earlier among tannery workers in Bangladesh (Hasan et al. 2016). It is important to treat these effluents that are discharged into the environment in order to reduce environmental pollution and the disease burden. Numerous technologies are available to mitigate Cr contaminated wastewater, which includes redox chemical processes (Franco et al. 2009), reverse osmosis, coagulation, and precipitation (Ahluwalia and Goyal 2007, Xu et al. 2018). However, these aforementioned methods are expensive and energy-consuming, and they have the risk of secondary chemical contamination. Wastewater and effluents with heavy metal contamination can be treated with potential microorganisms. These microorganisms can treat large volumes of effluent with a low operational cost and low energy demand yet performing a higher efficiency of metal removal (Liu et al. 2012, Thacker et al. 2007, da Rocha et al. 2018). Microorganisms provide higher surface area to volume ratio due to their small size and can assimilate metals from surrounding settings very quickly. Toxic Cr (VI) reduction to its nontoxic Cr (III) form is one of the mechanisms used by many organisms to survive in Cr (VI) contaminated effluents. The present study was conducted for the isolation and identification of Cr (VI) resistant bacteria from tannery effluents and polluted water and *in vitro* determination of their potential application in the removal of Cr (VI) from effluent water.

Materials and Methods

Area of sample collection: The study area comprised of the tannery area at Hazaribagh Thana which is situated on the south-western part of Dhaka city, Narayangang upazilla sadar and Shittolakkha river. Nine different sites were selected from Hazaribagh Tannery area to Rayerbazar sluice gate area, Buriganga, Narayangang Upzilla sadar and Shittolakkha (Fig. 1) for collection of tannery effluent from drain water. Sampling has been done twice from these sites in December, 2022 and January, 2023.

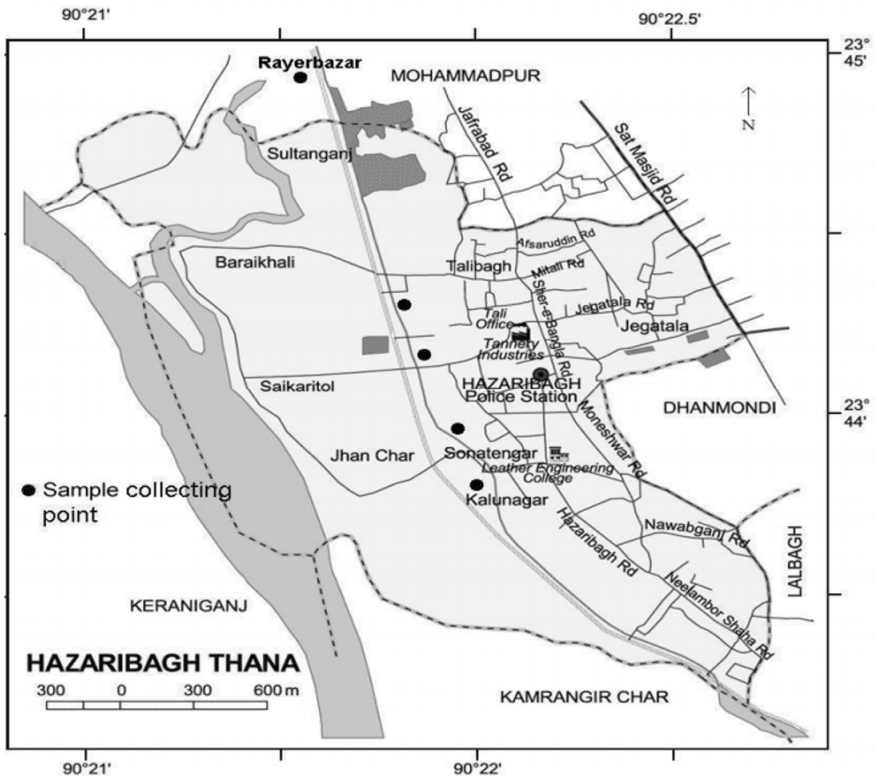

Fig. 1. Sampling sites within Dhaka city used in the present study.

Isolation of bacteria: A volume of 100 µl from each effluent/drain water sample was inoculated on Nutrient agar plates containing 100 mg/l Cr (VI) (as $K_2Cr_2O_7$) and incubated at 37°C for 24 hrs. Colonies growing on the plate were stored in glycerol broth at -20°C for further research.

Assay for Chromium (VI) tolerance: Tolerance of chromium (VI) (0 – 1000 µg/ml) by bacterial isolates was determined on nutrient agar plates. The maximum Cr (VI) concentration which supported the growth of the bacterial isolates was defined as the Maximum Resistance Level (MRL) (mg/l).

Cr (VI) reduction by whole cell in buffer: Cr reduction by whole cell in buffer was performed by following the method described by Ilias et al. (2011) with slight modification. Bacterial cells were grown in 50 ml Luria Bertani broth supplemented with 100 ppm Cr (as $K_2Cr_2O_7$) in a 250 ml Erlenmeyer flask. After 24h of incubation at 37°C and 150 rpm agitation, cells were harvested from 1.5 ml culture by centrifugation at 10,000 rpm for 5 min. The cell pellet was taken in an eppendorf tube and was washed twice with phosphate buffer followed by resuspension in 0.5 ml phosphate buffer (100 mM, pH 7.0). To initiate Cr (VI) reduction, 0.5 ml of $K_2Cr_2O_7$ was added (2.5 mg/l; 5 mg/l; 10 mg/l; 25 mg/l; 50 mg/l and 100 mg/l) into the cell suspension in phosphate buffer and incubated at 37°C in a water bath for 45 min. The reaction mixture was centrifuged at 10,000 rpm for 5 min and the supernatant was analysed for residual Cr (VI) determination. Residual hexavalent chromium was determined colorimetrically with a spectrophotometer following the S-diphenylcarbazide (DPC) method (Bartlet and James 1996). To determine the amount of Cr (VI) in the sample, 125 µl of the DPC reagent was added to 1 ml of chromium samples, mixed gently and kept at room temperature for 20 min to complete the reaction. Presence of pink colour indicated a positive result for reduction. The absorbance was measured at 540 nm using a spectrophotometer. A reaction blank was prepared by adding 0.5 ml phosphate buffer to 0.5 ml of $K_2Cr_20_7$ (2.5 mg/l, 5 mg/l; 10 mg/l; 25 mg/l; 50 mg/l; 100 mg/l) which was considered as 100% Cr (VI). Phosphate buffer was used to calibrate the spectrophotometer to zero.

Biosorption studies: Similar amount of living and dead cells were mixed separately in 10 ml of the metal solutions in a flask. The flasks were agitated on a shaker (150 rpm) at room temperature for 24 hrs. The samples were centrifuged at 10,000 × *g* for 20 min and supernatant liquid was used to estimate metal ion concentrations. Sorption of chromium (VI) was obtained by batch equilibrium method. Sorption experiments were performed in 100 ml of Cr (VI) solution in Erlenmeyer flasks. Biomass of bacterial strain was exposed to Cr (VI) solutions for 24 hours at 160 rpm/minute on orbital shaking incubator. Centrifugation at 5724 g for 15 minutes was used to separate biomass of strain from supernatant. The supernatant was then assayed for residual Cr (VI) by UV-VIS spectroscopy at 540 nm.

Cr (VI) sorbed by biosorbent was calculated using the following formula:

$$Q = V(Ci - Cf)\, M,$$

where Q = Metal ion [Cr (VI)] uptake capacity (mg/g), Ci = initial rate of metal in solution before sorption determination (mg/g), Cf = final concentration of metal in solution after the sorption determination (mg/g), M = Amount of biosorbent (g), V = solution volume (l). Difference in the initial and final Cr (VI) concentration is the metal adsorbed to the biosorbent.

Molecular identification by 16s rDNA sequencing: 16s rDNA sequencing was carried out for identification of the isolated bacteria. A pure colony was selected from Nutrient Agar plate supplemented with 100 ppm chromium and inoculated into Nutrient Broth

containing 100 ppm chromium and incubated for 24 hours at 37°C. One ml of bacterial culture was used for DNA extraction methods. A DNA purification kit (Favorgen, Germany) was used following the instructions provided in the manual.

PCR: Amplification was performed in a 25 µl reaction volume for each isolate containing 2 µl of template DNA, 19.5 µl water, 12.5 µl master mix (NEB, UK), 0.5 µl $MgCl_2$, 0.5 µl DMSO, 1.25 µl of each primer (forward and reverse). The PCR tubes were then transferred to a DNA thermal cycler for amplification of DNA. Universal 16s rDNA primers, 27 forward and 1492 reverse primers were used (Table 1).

Table 1. Universal primers used in PCR reaction.

Primer for 16s rDNA	Sequence	Amplicon size
27F	AGA GTT TGA TCM TGG CTC AG	1465bp
1492R	CGG TTA CCT TGT TAC GAC TT	

The PCR cycle included initial denaturation at 94°C for 10 minutes, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 45 seconds, extension at 72°C for 2 minutes finally ending in extension at 72°C for 10 min.

Agarose gel electrophoresis: Agarose (1.5%) was dissolved in TAE (Tris-acetate-EDTA) electrophoresis buffer (1X TAE buffer). Electrophoresis was carried out at 70V and 0.03A. The gel was viewed by UV trans-illuminator (Gel Doc, Alpha imager mini, USA) and the photograph was taken.

Results and Discussion

In recent years, microorganisms capable of removing toxic heavy metals from polluted water have gained attention and is an important area of research globally. The aim of this study was to isolate bacteria capable of chromium bioremediation from the polluted environment.

The present study was successful at isolating 71 bacterial colonies that grew in the presence of 100 ppm chromium. Tolerance of chromium was observed at different levels by the isolates (Fig. 2). All isolates grew in the presence of 100 ppm chromium, 93% in the presence of 200 ppm chromium, 46% in the presence of 400 ppm chromium, 6% in presence of 600 ppm chromium and 4% in presence of 1000 ppm chromium.

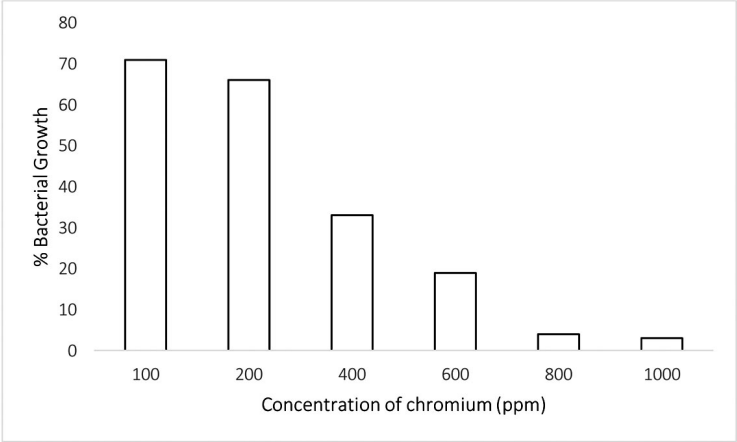

Fig. 2. Tolerance of chromium by isolated bacteria.

Fourteen representative isolates were tested for their ability to reduce chromium (VI). Isolated bacteria demonstrated reduction in the range of 23-89% (Fig. 3). Two bacteria which showed greater than 80% reduction are proposed as potential candidates for environmental bioremediation.

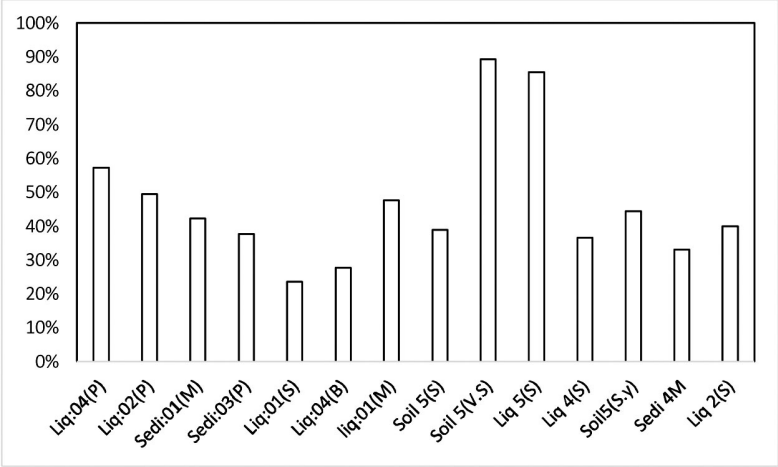

Fig. 3. Reduction of chromium determined by Diphenyl Carbazide Assay.

Decrease in concentration of chromium (VI) can occur either by adsorption onto bacterial cell surface or by reduction into chromium (III). Five isolates were chosen based on chromium reduction data and high tolerance. Equal volumes of the same culture were inoculated into media containing chromium (VI). One set contained culture that was autoclaved first to kill the cells. These dead cells will only be able to adsorb chromium (VI) without reducing it. The other set contained an equal volume of live cells that would

be able to both adsorb and reduce the metal. The concentration of chromium (VI) following incubation for an hour was determined by Diphenyl Carbazide Assay (Fig. 4). One of the isolates tested exhibited significant sorption of about 69%.

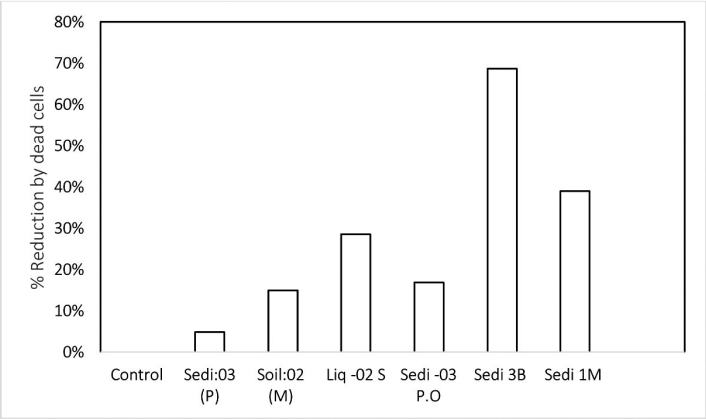

Fig 4. Sorption assay using dead cells for chromium binding

In the present study the universal primers (UniForward and UniReverse primers) were used for the amplification of 16S rDNA ribosomal smaller subunit. Resolution of PCR products by 1.5% agarose gel electrophoresis exhibited a distinct band of ~1kb amplicon for each candidate bacteria. A total of 6 bacterial 16rDNA sequences were obtained during this study period (Fig. 5).

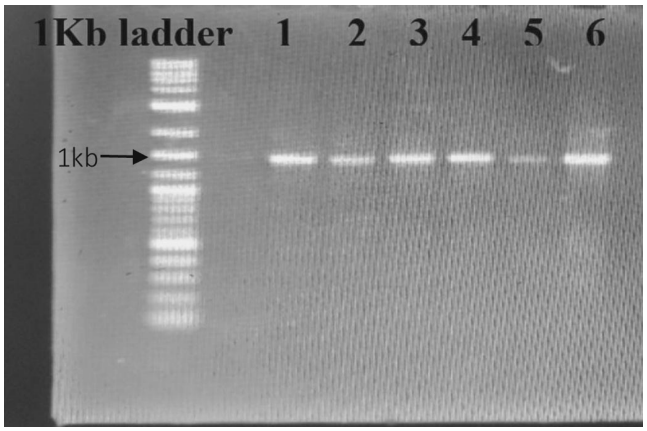

Fig. 5. Agarose gel electrophoresis image of 16S rDNA amplification products.

The identity of the six isolates are shown in Table 2. *Kurthia gibsonii* was identified as one of the isolates capable of chromium bioremediation. In a study by Niveshika et al. (2016), *Kurthia gibsonii* was also reported as a bacterial species capable of chromium bioremediation.

Table 2. Molecular Identification of selected bacterial isolates

Serial of isolate	Identified species	% similarity	E- value
Isolate 1	*Kurthia gibsonii*	100	0
Isolate 2	*Acinetobacter schindleri*	100	0
Isolate 3	*Pseudomonas aeruginosa*	100	0
Isolate 4	*Exigubacterium profundum*	100	0
Isolate 5	*Rhodococcus* sp.	100	0
Isolate 6	*Bacillus* sp.	100	0

Acinetobacter schindleri was also identified as another potential candidate for bioremediation. In a previous study, Srivasta and Shekhar (2007), reported that *Acinetobacter* sp. isolated from pulp and paper mill consortium removed higher amount of chromate [Cr(VI)] under aerobic conditions. Parameters optimized in different carbon, nitrogen sources, and pH, indicated maximum removal of chromate in sodium acetate (0.2%), sodium nitrate (0.1%) and pH 7 by *Acinetobacter* sp. Bacteria was applied in 2-l bioreactor significantly removed chromate after 3 days. The results of the study indicated removal of more than 75% chromium by *Acinetobacter* sp. determined by diphenylcarbazide colorimetric assay and atomic absorption spectrophotometer after 7 days. In comparison with the present study, incubation of potential bacteria with chromium for 7 days enabled 83% reduction. However, conditions for bioremediation were not optimized in this study. In another study, Panda and Sarker (2012) reported that *Acinetobacter* sp. PD 12 (NCBI GenBank USA Accession no. GU084179) showed powerful chromium resistivity and bioremediation capabilities among many stains isolated from tannery waste.

In a number of studies, Gram negative bacteria have been found to be associated with heavy metal bioremediation. In this study, a third potential chromium bioremediation candidate was identified as *Pseudomonas aeruginosa*. *Pseudomonas* species have been isolated by various researchers worldwide that have heavy metal bioremediation potential (Tasleem et al. 2023, Deepali 2011). In a published literature, Chunxi Kang et al. (2014) reported that the potential role of parameters in the reduction of hexavalent chromium [Cr(VI)] by *Pseudomonas aeruginosa* is not well documented. In their study, laboratory batch studies were conducted to investigate the effect of environmental factors. Heavy metal tolerant *Pseudomonas* was isolated from industrial effluent by Nath et al. (2012). Based on these published information, *Pseudomonas aeruginosa* isolated in this study could be a potential candidate for bioremediation.

In the present study, *Exigubacterium* sp. was identified as a potential candidate for chromium (VI) removal. In a previous study (Gupta et al. 2012), *Exiguobacterium aestuarii* strain CE1, displayed high Cr (VI) removal capabilities. *Exiguobacterium profundum* strain KSKE41, has been identified to tolerate the stress of other heavy metals

(cadmium, mercury, copper, zinc, arsenic, and manganese) to variable extent along with chromium (VI) (Rizvi et al. 2016). In another study, *Exiguobacterium indicum* has been reported to resist exceptionally high concentrations of hexavalent chromium (1500 mg/l) (Mohapatra et al. 2017). The *Exigubacterium* sp. isolated in the present study can be tested further for chromium bioremediation under different conditions for environmental pollutant removal in the future.

Rhodococcus sp. also showed promising result for chromium bioremediation in this study. In 2000, a novel species named as *Rhodococcus pyridinivorans* was found to efficiently degrade various toxic compounds in the environment (Yoon et al. 2000).

The identification of *Bacillus* sp. with chromium bioremediation potential in this study is supported by widespread published literature (Thatoi et al. 2014).

Development of process with combination of biological and chemical methods that would concomitantly reduce Cr (VI) by enzyme treatment as well as precipitate Cr (III) by chemical reaction could make the products chromium free in larger part. Microbial remediation through reduction of Cr (VI) is of enormous use both for effluent treatment before disposal in the tanning industries as well as for treating the SCW produced feed stuff to turn it into safer input. Because Cr (VI) is totally undesirable in health perspective. The identified isolates in the present study, may prove to successfully reduce Cr (VI), which can be used for the development of bioremediation process. However, for industrial adoption of remediation process, studies are required on feasible production of the reductase enzyme or direct reduction of Cr (VI) by the organisms in whole cell procedure to provide economic and rapid application.

This study has successfully isolated six bacterial strains which can tolerate high concentrations of chromium and reduce toxic hexavalent chromium to non-toxic form. Both adsorption and reduction abilities to varying extents were demonstrated by the selected isolates. Whole Genome Sequence analyses will help to identify specific genes involved with bioremediation. Further investigations are necessary to understand their mechanism of tolerance to other heavy metals. Strain development using genetic engineering can be applied to improve their heavy metal bioremediation potential in future studies.

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