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Cr (VI) Reduction by Indigenous *Bacillus* Species PB5 Isolated from Contaminated Soil of Abeokuta Ogun State, Nigeria

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ABSTRACT

Chromium (VI) contamination has accelerated due to rapid industrialization worldwide. Aim of this study is to check the bacterial species for their tolerance towards chromium (VI), chromium (VI) reduction under various pH and further check whether these bacteria are reducing Cr (VI) under the influence of various metals, carbon source and protein denaturant. Bacterial strains were isolated from metal contaminated soils of Abeokuta. All of the isolates showed tolerance to chromium (VI). Among all the strains, only *Bacillus* species PB5 showed reduction of Chromium (VI). Maximum reduction (90%) of chromium (VI) was observed at pH 7 by *Bacillus* species PB5. Similarly, *Bacillus* species PB5 also reduced the chromium considerably at pH 6 (86%) and pH 8 (87.5%) at a concentration of 100 $\mu\text{g Cr mL}^{-1}$ after 120 h of incubation. *Bacillus* species PB5 also showed chromium (VI) reduction under various metals, protein denaturant and carbon source. There was maximum decrease in Cr (VI) reduction under the influence of PbCl_2 which was followed by ZnCl_2 . Urea and citrate also decreased Cr (VI) reduction compared to control cells. Due to above properties strains could therefore be used as bioremediators of metals in soils contaminated with heavy metals.

Key words: *Bacillus* species, Chromium (VI) tolerance, antibiotic resistance, chromium (VI) reduction, carbon source, metals

INTRODUCTION

The contamination of chromium (VI) is mainly is due to the use of Cr (VI) in leather, tanning, metallurgy, electroplating, textile and pigment manufacturing industries (Wang and Xiao, 1995; Pattanapitpaisal *et al.*, 2001a; Sultan and Hasnain, 2007). Chromium occurs either in trivalent or hexavalent which affect growth of microorganisms present in the environment (Ortegel *et al.*, 2002). Hexavalent chromium is highly soluble in water, permeable through biological membranes and interacts with proteins and nucleic acids which makes it more toxic and carcinogenic than trivalent (Kamaludeen *et al.*, 2003; Ackerley *et al.*, 2006). Reduction of Cr (VI) leads to the formation of stables, less soluble and less toxic Cr (III). Reduction of toxic Cr (VI) to Cr (III) is thus a useful process for remediation of Cr (VI) affected environments (Jeyasingh and Philip, 2005). The reduction/ detoxification of Cr (VI) by microbes is, however, inexpensive and environmentally safe approach and provides a viable option to protect the environment from chromium toxicity. The

reduction of Cr (VI) is reported in *Bacillus* (Nguema *et al.*, 2014; Elangovan *et al.*, 2006; Chaturvedi, 2011), *Pseudomonas* sp. (Wani and Ayoola, 2015; Rahman *et al.*, 2007), *Escherichia coli* (Bae *et al.*, 2005), *Microbacterium* (Pattanapitpaisal *et al.*, 2001b), *Ochrobactrum intermedium* (Faisal and Hasnain, 2005) and *Micrococcus* (Sultan and Hasnain, 2005).

Detoxification of chromium by microbes may occur directly or indirectly and is affected by pH, chromate concentration, incubation periods and the types of microbes involved. In the direct mechanism, microbes enzymatically (chromium reductases) reduce chromium (Soni *et al.*, 2013; Losi *et al.*, 1994) while in the indirect method, reductants or oxidants, such as H₂S, reduce chromium (DeFilippi and Lupton, 1992). Furthermore, in growing cultures with added carbon sources as electron donors and in cell suspensions, Cr (VI) reduction can be predominantly aerobic or anaerobic but generally not both. Interestingly, chromium reductases can catalyse reduction of Cr (VI) to Cr (III) anaerobically (Lovely and Phillips, 1994), aerobically (Cervantes *et al.*, 2001) and also both anaerobically and aerobically (Marsh and McInerney, 2001). The Cr (VI) reductase may be present in the membrane fraction of the cells of PGPR, as found in *Pseudomonas fluorescens* and *Enterobacter cloacae* (Wang *et al.*, 1990) and *Arthrobacter* species (Dey and Paul, 2013). The reduction of Cr (VI) to Cr (III) results in the formation of insoluble precipitate [Cr(OH)₃], which is easily removed from wastewater (Jeyasingh and Philip, 2005). The enzyme chromium reductase found in *P. ambigua* (Campos-Garcia *et al.*, 1997) and *Bacillus* sp. (Wang *et al.*, 1991) were purified and characterized. More recently, to clone a chromate reductase gene, novel soluble chromate reductase of *P. putida* was purified to homogeneity and characterized (Puzon *et al.*, 2002). The reductase activity was NADH- or NADPH-dependent. Reduction of Cr (VI) by H₂S produced by the bacterial cells is found in soil environments which are rich in sulfate under anaerobic conditions (Losi *et al.*, 1994). Hydrogen sulfide, produced in acid sulfate soil under reducing conditions, is easily precipitated as FeS in reduced soils (Eary and Rai, 1991) and sediments. Fe (II) and H₂S, both microbially produced, are effective reductants of Cr (VI) under reduced conditions as is the FeS (Karnachuk, 1995). Present study was therefore under taken to check the chromium (VI) reduction ability of the bacterial species under the influence of different environmental factors.

MATERIALS AND METHODS

Collection of soil sample: The soil samples for the isolation of Chromium (VI) resistant bacteria were collected from the contaminated soils of Abeokuta, Ogun state, Nigeria. Abeokuta is situated at 7.15° North latitude, 3.35° East longitude and 67 m elevation above the sea level.

Isolation of bacteria: Bacteria were isolated from the contaminated soil of Abeokuta on nutrient agar medium by spread plate technique (Holt *et al.*, 1994). One milliliter of water sample was added to a flask containing 100 mL of normal saline solution and was serially diluted. A 10 µL of each suspension was spread plated on solid nutrient agar. Plates were incubated at 28±2°C for 24 h and the bacterial colonies were then purified and preserved on nutrient agar slants for further experiments.

Morphological and biochemical characterization of bacterial species: Morphological and biochemical characterization of the bacterial isolates were performed according to the Bergey's manual (Holt *et al.*, 1994). Characteristics of catalase, urease, oxidase, starch hydrolysis, gelatin liquefaction, Voges-Proskauer (V.P.), Methyl Red (M.R.), indole production, H₂S production and the utilization of carbon/nitrogen sources were tested.

Evaluation of bacterial strains for chromium (VI) tolerance: The isolated bacterial strains were tested for their sensitivity/resistance to chromium (VI) by agar plate dilution method Holt *et al.* (1994) using nutrient agar. The freshly prepared agar plates amended with increasing concentration of chromium (0-1000 $\mu\text{g mL}^{-1}$) were spot inoculated (10 μL) with 10^8 cells mL^{-1} . Plates were incubated at $28\pm 2^\circ\text{C}$ for 72 h and the highest concentration of heavy metals supporting growth was defined as the Maximum Resistance Level (MRL). Each experiment was replicated three times.

Determination of antibiotic resistance of the bacterial strains: To determine the resistance to antibiotics, the bacterial strains were tested for their sensitivity to thirteen different antibiotics. Their reactions to antibiotics were determined by the disc diffusion method. A 0.1 mL of the overnight grown broth culture was spread on the surface of nutrient agar. The antibiotic discs of known potency were then placed on the agar surface and then the plates were incubated at $28\pm 2^\circ\text{C}$ for 24 h. The zones of inhibition around the antibiotic discs were measured (in millimeter) against the following antibiotics that were used: tetracycline (10 μg), nitrofurantoin (300 μg), gentamycin (10 μg), Cloxacillin (5 μg), Cotrimoxazole (25 μg), Chloramphenicol (30 μg), Augmentin (30 μg), Amoxicillin (25 μg), Erythromycin (5 μg), Cefuroxime (30 μg), Ceftazidime (30 μg), Ciprofloxacin (5 μg), Nitrofurantoin (300 μg) and Ofloxacin (5 μg).

Chromium (VI) reduction by *Bacillus* species: To assess the effect of pH on hexavalent chromium [Cr (VI)] reduction *in vitro*, the Nutrient Broth (NB) was amended with 100 $\mu\text{g mL}^{-1}$ of Cr (VI) and the autoclaved medium was adjusted to pH 6, 7 and 8 with 1 M HCL or 1 M NaOH and incubated at $28\pm 2^\circ\text{C}$ for 120 h. For Cr (VI) reduction, 1 mL culture from each flask was centrifuged (6000 rpm) for 10 min at 10°C and Cr (VI) in the supernatant was determined by 1,5-diphenyl carbazide method (Eaton *et al.*, 1992) upto 120 h. Briefly, the test samples were acidified (pH 1-2) and 1,5-diphenyl carbazide (50 $\mu\text{g mL}^{-1}$) was added and Cr (VI) concentration was detected by UV-VIS spectrophotometer at 540 nm.

Effect of metal ions, electron donors and protein denaturants on Cr (VI) reduction: To assess the effect of (0.1 mM each) metal ions (CuCl_2 , ZnCl_2 and PbCl_2), electron donor (citrate) protein denaturants (urea) on hexavalent chromium [Cr (VI)] reduction *in vitro*, the Nutrient Broth (NB) was amended with 100 $\mu\text{g mL}^{-1}$ of Cr (VI) and CuCl_2 , ZnCl_2 , PbCl_2 , citrate and urea. The autoclaved medium was adjusted to pH 7 with 1 M HCL or 1 M NaOH and incubated at $28\pm 2^\circ\text{C}$ for 120 h. After incubation, the bacterial culture solutions were centrifuged (6,000 rpm⁻¹ for 20 min at 4°C) and Cr(VI) concentration was measured as described above.

Data of three replicates were subjected to statistical analysis using sigma plot 12.0. The values indicate the Mean \pm SD of three replicates.

RESULTS AND DISCUSSION

Characterization of bacterial isolates: All the bacterial isolates were characterized based on morphological and biochemical tests and were all found to be Bacilli, gram negative, oxidase positive, urease positive, catalase positive, citrate positive and MRVP test positive. They also utilized different carbon sources. Based on the above properties strain PB1, PB3, PB4 and PB5 were characterized as *Bacillus* spp. whereas PB2 *Staphylococcus* spp. and PB6 as *Klebsiella* spp.

Evaluation of bacterial for Cr (VI) tolerance: In this study all the bacterial strains were checked for their resistance to Cr (VI) by agar plate dilution method (Fig. 1). Generally, bacterial strains showed a varied level of tolerance to Cr (VI). Among the bacterial strains, *Klebsiella* spp. PB6 and *Bacillus* spp. PB 5 showed highest tolerance to Chromium (VI) at a concentration of 1000 $\mu\text{g mL}^{-1}$. There are reports which showed the resistance of bacteria to Cr (VI) (Wani *et al.*, 2015; Wani and Khan, 2013). There are many reports which showed varied level of tolerance by bacteria. This varied level of resistance could be due to the variation in growth conditions employed (Rajkumar *et al.*, 2005). For example, *Intrasporangium* sp. Q5-1 has shown a tolerance level of 17 mM to Cr (VI) (Yang *et al.*, 2009) while as *Bacillus* spp. PZ3 and *Streptococcus* spp. The PZ4 showed highest tolerance to Chromium (VI) at concentration of 700 $\mu\text{g mL}^{-1}$ (Wani *et al.*, 2015).

Resistance of the bacterial isolates to antibiotics: Resistance to antibiotics among different isolated metal-tolerant bacterial strains differed considerably (Table 1). Among the isolated bacterial isolates, 100% were resistant to ciprofloxacin and ofloxacin, 83.33% to Nitrofurantoin, Erythromycin, Cefuroxime and Ceftazidine, whereas 66.66% strains to Tetracycline. No strain was found to be resistant to Augumentin and Gentamycin (Table 1).

Effect of pH on Cr (VI) reduction: Chromium is an environmental pollutant released from various industries including tanneries, metal cleaning and processing, chromium plating, wood

Table 1: Resistant pattern of bacterial species to various antibiotics

Antibiotics used	Concentrations ($\mu\text{g mL}^{-1}$)	No. of resistant isolates (%)
Tetracycline	10	4 (66.66)
Cloxacillin	5	2 (33.33)
Chloramphenicol	30	1 (16.66)
Nitrofurantoin	200	5 (83.33)
Augumentin	30	0 (0.00)
Gentamycin	10	0 (0.00)
Cotrimoxazole	25	3 (50.00)
Ampicillin	25	2 (33.33)
Erythromycin	5	5 (83.33)
Cefuroxime	30	5 (83.33)
Ceftazidine	30	5 (83.33)
Ciprofloxacin	5	6 (100.00)
Ofloxacin	5	6 (100.00)

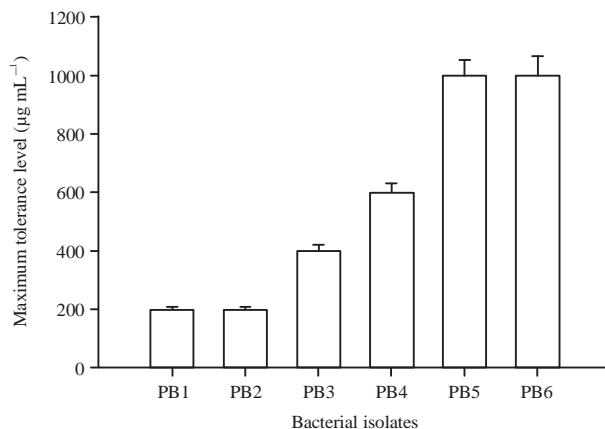


Fig. 1: Maximum tolerance level of different bacterial isolates to chromium (VI)

processing and alloy formation. Chromium [Cr (VI)] is the most toxic and carcinogenic (Kamaludeen *et al.*, 2003) due to its high solubility, rapid permeability and their intracellular proteins and nucleic acids (Reeves *et al.*, 1983). The reduction of Cr (VI) leads to the formation of stable, less soluble and less toxic Cr (III) and is thus, a useful process for remediation of Cr (VI) affected environments (Thacker *et al.*, 2007). Thus detoxifications of chromium by bacterial strains is thus a good technique to clean the environment from chromium. Therefore, the present study was designed to determine the Cr (VI) reducing ability of the metal tolerant strains.

Among all the strains, only one strain *Bacillus* spp. PB5 showed chromium reducing ability under *in vitro* conditions as this strain was highly resistant to chromium (VI). This study was carried out to access the effect of different pH values on the reduction of Cr (VI).

The effect of different pH values on the reduction of chromium (VI) is shown in Fig. 2. Maximum reduction (90%) of chromium (VI) was observed at pH 7 by *Bacillus* sp. PB5. Similarly *Bacillus* spp. PB5 also reduced chromium considerably at pH 6 (86%) and pH 8 (87.5%), respectively, at a concentration of 100 $\mu\text{g Cr mL}^{-1}$ after 120 h of incubation.

Present study is in correlation with the study of Yang *et al.* (2009) who also observed considerable reduction of chromium. Cr (VI) reduction under various pH and different concentrations of Cr (VI) was also observed by Wani *et al.* (2015), who observed maximum reduction of Cr (VI) at pH 7.0 and 100 $\mu\text{g mL}^{-1}$ of chromium.

Effect of metal ions, electron donors and protein denaturants on Cr (VI) reduction: The effect of different metal ions, electron donor and protein denaturant on the reduction of chromium (VI) is shown in Fig. 3. Maximum effect on Cr (VI) reduction by *Bacillus* sp. PB5 was shown by PbCl_2 , followed by ZnCl_2 whereas less effect was shown by CuCl_2 . Citrate and urea also showed decrease in chromium reduction but urea showed more effect on reduction as compared to citrate. This study has concluded that nutrient broth is best for Cr (VI) reduction in comparison to citrate. Metal ions have been known to affect chromate reductase activity. Ohtake *et al.* (1990) reported that Cu^{2+} inhibit the chromate reductase activity of *Enterobacter cloacae* whereas Park *et al.* (2000) found reduction in soluble chromate reductase activity in *Pseudomonas putida*. In another study Pal *et al.* (2013) also found reduction in soluble chromate reductase activity in *B. sphaericus* AND 303. Metal ions may affect microbial Cr (VI) reduction in two ways: destruction of cells (decrease in cell growth) and inhibition of enzymes responsible for Cr (VI) reduction. Metal ions may absorb on to cell walls or complex with enzymes responsible for Cr (VI) reduction.

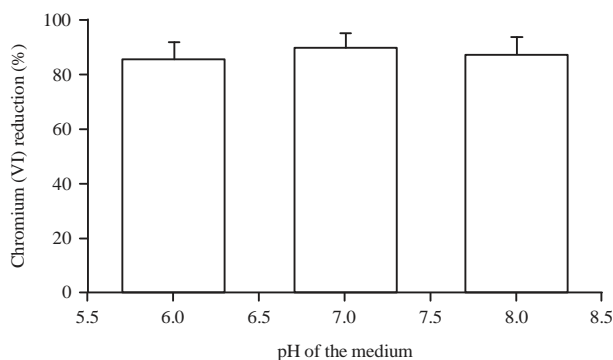


Fig. 2: Effect of pH on Cr (VI) reduction ability of *Bacillus* species PB5 after 120 h of growth in nutrient broth

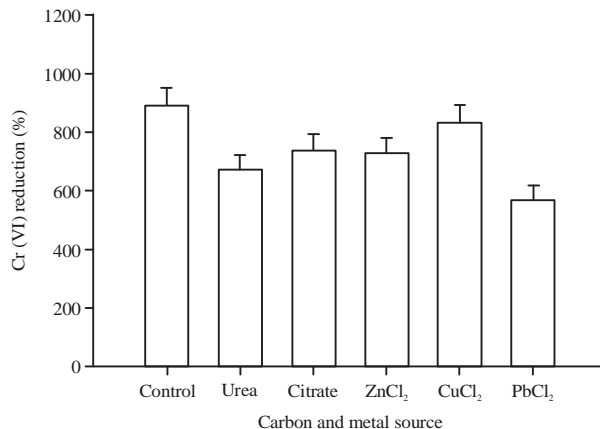


Fig. 3: Effect of carbon, protein denaturant and metals on Cr (VI) reduction by *Bacillus* species PB5 after 120 h of growth in nutrient broth

Citrate has shown decrease in the Cr (VI) reduction compared to control. It has shown a Cr (VI) reduction of 74% and decreased Cr (VI) reduction by 16% compared to control. Similar decrease in Cr (VI) by citrate compared to control has been shown by Soni *et al.* (2013). In another study Yang *et al.* (2009) found decrease in chromium (VI) reduction by NADH, methanol and ethanol compared to control. In this study we found decrease in Cr (VI) reduction by acetate possibly because Cr (VI) reductase enzyme of strain PB5 is not acetate dependent. Urea has shown decrease in Cr (VI) reduction compared to control. Similar decrease in Cr (VI) reduction by urea has been studied by Soni *et al.* (2013).

CONCLUSION

This study concludes that the bacterial strain not only tolerated metal ions and antibiotics but also reduced chromium (VI) under different pH, protein denaturant, carbon source and metals. Due to multifarious properties expressed by the bacterial strain, this strain could therefore be used as bioremediator of metals in soils contaminated with heavy metals. Therefore, the findings of this study would help in developing appropriate conditions for the treatment of contaminated effluents/soils. In future we can study the role of chromium reductase enzyme for the reduction of Cr (VI) to Cr (III).

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