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CR (VI) Removal by Indigenous *Klebsiella* Species PB6 Isolated from Contaminated Soil under the Influence of Various Factors

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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 *Klebsiella* spp. PB6 showed reduction of chromium (VI). Maximum reduction (89.5%) of chromium (VI) was observed at pH 7 by *Klebsiella* spp. PB6. Similarly, *Klebsiella* spp. PB6 also reduced the chromium considerably at pH 6 (88%) and pH 8 (71%) at a concentration of 100 μg Cr mL⁻¹ after 120 h of incubation. *Klebsiella* spp. PB6 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 ZnCl₂ which was followed by PbCl₂. 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:** *Klebsiella* species, chromium (VI) tolerance, chromium (VI) reduction, carbon source, metals

**INTRODUCTION**

The contamination of chromium (VI) is mainly due to the use of Cr (VI) in leather, tanning, metallurgy, electroplating, textile and pigment manufacturing industries (Wang and Xiao, 1995; Pattanapipitpaisal *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 it 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 stable, 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 Phillip, 2005; Desai *et al*., 2008). 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) has been reported in *Bacillus* (Elangovan *et al*., 2006; Chaturvedi, 2011), *Pseudomonas* sp., (Wani and Ayoola, 2015; Rahman *et al*., 2007),
Escherichia coli (Bae et al., 2005), Microbacterium (Pattanapipitpaisal et al., 2001b), Ochrobactrum intermedium (Faisal and Hansnain, 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 (Losi et al., 1994; Dey and Paul, 2013) while in the indirect method, reductants or oxidant, 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 catalyze reduction of Cr (VI) to Cr (III) anaerobically (Lovley and Phillips, 1994), aerobically (Cervantes et al., 2001) and also both anaerobically and aerobically (Marsh and Mclnerney, 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). The reduction 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), Bacillus sp., (Wang et al., 1991) and Bacillus cereus Pf-1 (Nguema et al., 2014) 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. The 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 undertaken to (1) determine the tolerance pattern of bacteria to Cr (VI), (2) check chromium (VI) reduction by free cells under varying pH (3) check chromium reduction by bacterial cells under the influence of various metals, carbon source and protein denaturant.

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.

Isolation of bacteria: Bacteria were isolated from the contaminated soil of Abeokuta on nutrient agar medium by spread plate technique. One gram of soil sample was added to a flask containing 9 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.

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 (0-700 μg mL⁻¹) were spot inoculated (10 μL) with 10⁸ cells mL⁻¹. Plates were incubated at 28±2°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.
Chromium (VI) reduction by *Klebsiella* spp: To assess the effect of pH on hexavalent chromium [Cr (VI)] reduction *in vitro*, the Nutrient Broth (NB) was amended with 100 µg mL⁻¹ of Cr (VI) and the autoclaved medium was adjusted to pH 5, 6, 7, 8 and 9 with 1 M HCL or 1 M NaOH and incubated at 28±2°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 µg 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 (CuCl₂, ZnCl₂ and PbCl₂), electron donor (citrate) protein denaturants (urea) on hexavalent chromium [Cr (VI)] reduction *in vitro*, the Nutrient Broth (NB) was amended with 100 µg mL⁻¹ of Cr (VI) and CuCl₂, ZnCl₂, PbCl₂, citrate and urea. The autoclaved medium was adjusted to pH 7 with 1 M HCL or 1 M NaOH and incubated at 28±2°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.

Statistical analysis: Data of three replicates was subjected to statistical analysis using sigma plot 12.0. The values indicate the Mean±SD of three replicates.

RESULTS AND DISCUSSION

Evaluation of bacterial strains for Cr (VI) tolerance: The bacterial strains were evaluated for their tolerance to various concentrations of chromium (VI) using 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 the highest tolerance to chromium (VI) at a concentration of 1000 µg mL⁻¹. There are reports which have shown the resistance of bacteria to Cr (VI) (Wani and Khan, 2013). There are many reports which have shown 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

Fig. 1: Maximum tolerance level of different bacterial isolates to chromium (VI)
has shown a tolerance level of 17 mM to Cr (VI) (Yang et al., 2009) while as Bacillus spp., PZ3 and Streptococcus spp. PZ4 showed highest tolerance to chromium (vi) at concentration of 700 μg mL⁻¹ (Wani et al., 2015). The metal tolerant strains were characterized by physiological, morphological and biochemical characteristics. The strain PB1, PB3, PB4 and PB5 were characterized as Bacillus spp. PB2 as Staphylococcus spp. while as PB6 was characterized as Klebsiella spp.

**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 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 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 Klebsiella spp. PB6 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 (89.6%) of chromium (VI) was observed at pH 7 by Klebsiella spp. PB6. Similarly Klebsiella spp. PB6 also reduced the chromium considerably at pH 6 (88%) and pH 8 (71%) respectively, at a concentration of 100 μg Cr mL⁻¹ after 120 h of incubation.

Our study is in correlation with the study of Yang et al. (2009), who also observed considerable reduction of chromium. The 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 μg mL⁻¹ of chromium. Reduction has also been observed by Soni et al. (2013).

![Fig. 2: Effect of pH on Cr (VI) reduction ability of Klebsiella spp. PB6 after 120 h of growth in nutrient broth](image-url)
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 the reduction by *Klebsiella* spp. PB6 was shown by ZnCl$_2$, followed by PbCl$_2$ whereas, less effect was shown by CuCl$_2$. Citrate and urea also show decrease in chromium reduction but the reduction was still high which was more than 75%. 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*. 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. Citrate has shown decrease in the Cr (VI) reduction compared to control. It has shown a Cr (VI) reduction of 75% and decreased Cr (VI) reduction by 14.5% 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 PB6 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

Based on the novel properties of chromium resistance and reduction, this bacterial strain could be used for Cr (VI) remediation in chromium contaminated soils.

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