JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Dec. 2014. Vol. **8**(6), p. 4289-4295

Detoxification of Carcinogenic Cr (VI) by Combined Action of *Bacillus pumilus*-S4 and *Pseudomonas doudoroffii*-S5 in Associated with Hydrophytes

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(Received: 26 June 2014; accepted: 21 August 2014)

Two chromium resistant bacterial strains (*Bacillus pumilus*-S4 and *Pseudomonas doudoroffii*-S5) isolated from tanneries effluent of were reported in this study. Strains resist up to 20 mg K_2CrO_4 ml^{-1} on nutrient agar and 2.2 mg ml^{-1} in acetate minimal medium. Chromate uptake by heat killed cells of *Bacillus pumilus*-S4 was (5.3 mg g^{-1} dry wt) 90 min as compared to living cells (25.4 mg g^{-1} dry wt). *Pseudomonas doudoroffii*-S5 reduced 14.2 mg Cr (VI) 36 h^{-1} at an initial concentration of 200 μg K_2CrO_4 ml^{-1}. Both strains also release extra cellular proteins in the medium which results extra cellular Cr (VI) detoxification. Cr (VI) removal efficiency of these strains was accelerated by *Pistia stratiotes* and *Eichornia crassipes* which provide energy source to strains. Hence these strains could be utilized for the treatment of chromium polluted industrial wastewater in association with metal hyperaccumulator hydrophytes.

Key words: *Bacillus pumilus*; Chromium; *Eichornia crassipes*; heavy metals; *Pistia stratiotes*; *Pseudomonas doudoroffii*.

Industrialization is although a premium for modern development, yet its effects on surroundings are equally hazardous[1]. Major waste water effluents such as antibiotics, drugs and heavy metals are carcinogenic, mutagenic and teratogenic[2]. Exposure or ingestion of contaminated food and water with heavy metals leads to metal intoxication[3]. Chromium is most toxic heavy metal that is generated from electroplating, leather tanning and in air craft industry[4]. Chromium occurs in many oxidation forms but the two important from are trivalent Cr (III) and hexavalent Cr (VI) chromium. Trivalent chromium is essential micronutrient in animals for proper glucose utilization[5]. Hexavalent chromium is mutagenic[6] and carcinogenic[7]. Conventional methods for treatment of toxic chromate include chemical reduction followed by precipitation, ion exchange, and adsorption on activated kaolinite, alum and ash[8]. Chemical detoxification of chromium is more expensive and poisonous for the environment. Biological methods of chromium removal are less expensive are environment friendly[9]. Detoxification of chromium with algae[10], fungi[11] plants[12] is well established. Many genera of bacteria have been reported to reduce Cr (VI) under either aerobic[13] or anaerobic[14] conditions. Beside this hydrophytes also play a significant role in metal removal via filtration, cation exchange, adsorption and through plant–induced chemical alteration in the root zone[15]. The free floating hydrophytes can be a better alternative technology for the extraction and removal of toxic chromium from contaminated waste water[16]. The present study focus on the

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utilization of chromium resistant bacteria and hydrophytes in combination for the removal of toxic chromium.

MATERIALS AND METHODS

Strains characterizations

Polluted samples were used for the isolation of chromium resistant bacteria. Strains were maintained at 4ºC on nutrient agar plates supplemented with 1000 µg ml^{-1} of K_2CrO_4. Temperature and pH were noted at the spot. Both these strains were characterized morphologically, biochemically, physiologically and genetically following Gerhardt *et al*[17].

Collections of hydrophytes

Free floating hydrophytes (*Pistia stratiotes* and *Eichhornia crassipes*) which were not previously exposed to heavy metals were collected from the Botanical Garden, Department of Botany, University of the Punjab, Lahore, Pakistan. Plant nurseries were maintained and equal sized plats were selected in the chromium removal experiments in combination with bacterial strains.

16S rRNA ribotyping

For the molecular taxonomic point of view, strains S4 and S5 which can resist K_2CrO_4, 16S rRNA gene sequencing was carried out. 16S rRNA gene (1300 bp) was amplified and the amplicon sequenced using fluorescent di-deoxy terminator cycle sequencing chemistry. The extension product was then separated on an ABI PRISM automated DNA sequencer and the data was compared to the MicroSeq® databases.

Chromate accumulation

For chromate accumulation experiments, both strains were grown in nutrient broth supplemented with two concentration of K_2CrO_4 (200 and 400 µg ml^{-1}). The inoculum was prepared as follow. i) One gram of pellet was taken and heat-killed at 121ºC for 15 minutes (Heat killed) and ii) same amount was used untreated (live cell mass). Inoculum was given in 250 ml flask and was incubated at 37°C. After different time incubation (30 min and 720 min), cells were harvested by centrifugation and washed thrice with autoclaved distilled water. Pellets obtained were digested and the total amount of accumulated chromium was determined by first oxidizing any trivalent chromium into hexavalent with $KMnO_4$ and analyzed spectrophotometrically at 540 nm in a spectrophotometer using diphenylcarbazide as the complexing agent[18]. All experiments were conducted in triplicate.

Cr (VI) reduction experiments

Bacterial Cr (VI) reduction was monitored at two different concentrations of K_2CrO_4 (200 and 400 µg ml^{-1}). The Cr(VI) reduction medium consist of following ingredients (grams per liter: Tryptone 10, yeast extract 5, NaCl 5, citric acid 1 and Na_2HPO_4 6.9)[19]. Medium was prepared in 250 ml flask and after sterilization the medium was supplied with 200 and 400 µg ml^{-1} of K_2CrO_4. Fresh inoculum was given in these flasks and incubated at 37ºC at 150 rpm. After 24 h, samples were withdrawn aseptically and were analyzed for Cr (VI) reduction. The analysis of Cr (VI) reduction by these strains was checked by using the classical spectrophotometeric method[18] using Diphenylecarbazide. The absorption was monitored at 540 nm.

Cr (VI) reduction by bacterial supernatant

To check the Cr (VI) reduction efficiency of bacterial supernatant, cell were grown and centrifuged at 10,000 x g for 10 min. The supernatant from 24 h old cultures were obtained and utilized both as such and after autoclaving (to denature the protein involved in Cr (VI) reduction). Initial K_2CrO_4 concentration used for this experiment was 200 and 400 µg ml^{-1}. The ratio of Cr (VI) reduction medium and supernatant was 1:1.

Cr removal by bacteria in combination with hydrophytes

To investigate the conjunctive effect of both plants and bacteria on the Cr removal ability, equal sized plants were selected and were exposed to an initial K_2CrO_4 concentration of 50 µg ml^{-1}. Bacterial inoculum was given from freshly prepared overnight cultures. Inoculum of 100 µl of both strains was added in the solution. The incubation was done at plant growth chamber with controlled light and temperature for 15 days. After 15 days incubation, plants were harvested and Cr concentration in both plants was assayed by utilizing oven dried plant material. Cr left in the supernatant and accumulated by bacterial strains was also analysed.

Statistical analysis

Standard errors of the means were calculated following Steel and Torrie[20].

RESULTS

Strains characterizations

Both these strains could resist high concentration of K_2CrO_4 both on nutrient agar (up to 20 mg ml^{-1}) and acetate minimal medium (2.2 mg ml^{-1}). Strain S4 is a gram positive aerobic spore forming rod while strain S5 is gram negative facultative anaerobic motile rod. The results of 16Sr RNA gene sequencing revealed that strains S4 and S5 identified as *Bacillus pumilus*-S4 and *Pseudomonas doudoroffii*-S5, respectively. Both strains cloud resist multiple heavy metals and antibiotics. The optimum temperature and pH for maximum growth of these strains is 37ºC and 7, respectively.

Cr accumulation

Fig. 1 revealed that Cr accumulation in these strains is metabolically linked. After 30 min of incubation, heat killed cells of strain *B. pumilus*-S4 accumulated 5.7 and 8.9 µg K_2CrO_4 g^{-1} dry weight, at an initial K_2CrO_4 concentration of 200 and 400 µg ml^{-1}, respectively. While in the same time period living cells of this strain accumulated 6.8 and 9.2 µg K_2CrO_4 g^{-1} dry weight (Fig. 1). But after 720 min of incubation, the amount of Cr

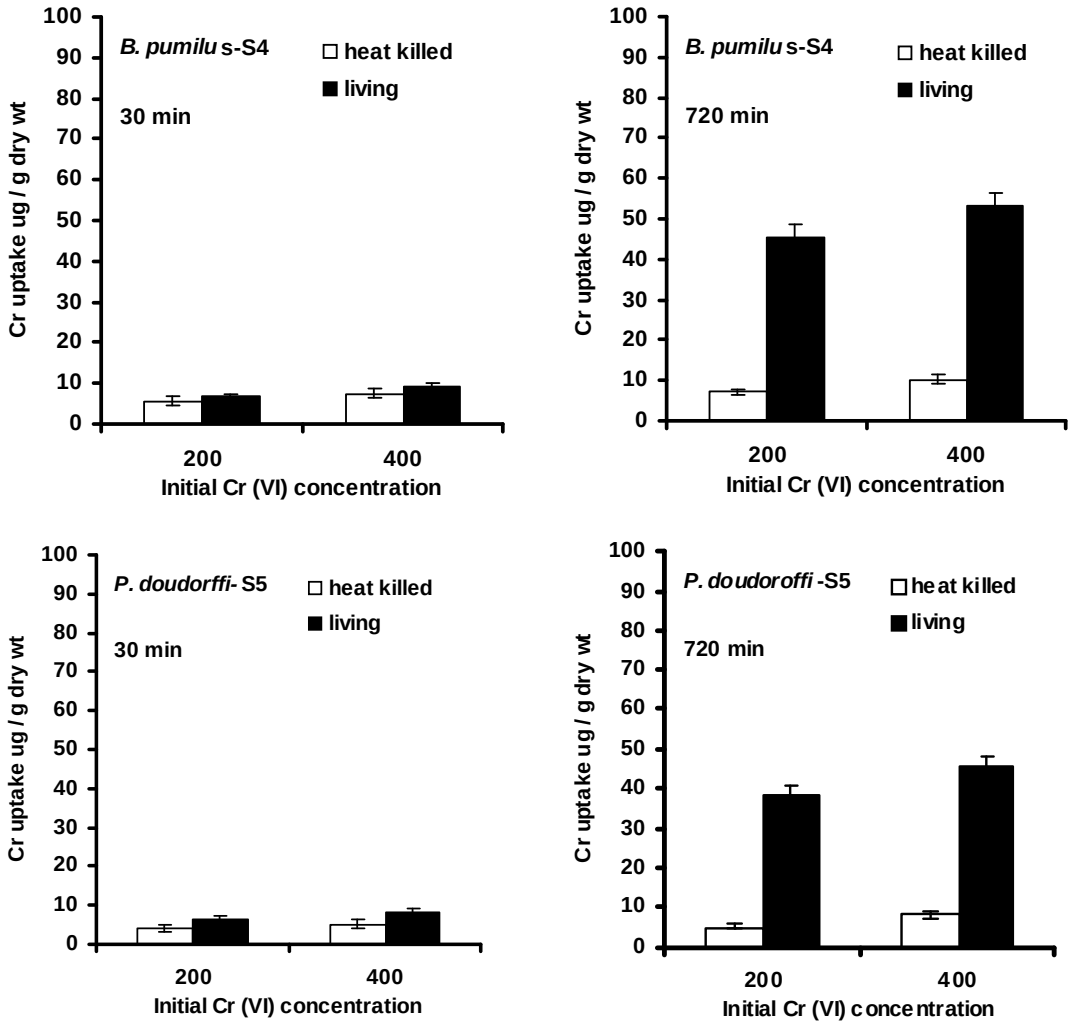

Fig. 1. Chromium accumulation potential of bacterial strains (*B. pumilus*-S4 and *P. doudoroffii*-S5) at different initial K_2CrO_4 concentrations of 200 and 400 µg ml^{-1} of K_2CrO_4 after contact time of 30 min and 720 min at 37°C and pH 7. Cells were used as heat killed and live

accumulated by living cells (45.6 µg K_2CrO_4 g^{-1} dry weight) was much higher (5 to 6 times) as compared to heat killed cells (7.2 µg K_2CrO_4 g^{-1} dry weight) (Fig. 1). Almost same trend was observed in case of strain *P. doudoroffii*-S5.

Cr (VI) reduction

Cr (VI) reduction in these strains was monitored at various initial Cr (VI) concentrations (200 and 400 µg ml^{-1}) and incubation times (12, 36 and 72 h). Strain *B. pumilus*-S4 reduced showed 28, 69 and 85% of Cr (VI) after 12, 36 and 72 h, respectively, at an initial K_2CrO_4 concentration of 200 (Fig. 2). At higher initial Cr (VI) concentration (400 µg ml^{-1}), the reduction %age decreased but overall more amount of chromate was reduced after 72 h of incubation (Fig. 2). After 72 h of incubation, strain *B. pumilus*-S4 reduced 64% while strain *P. doudoroffii*-S5 reduced 52% of Cr (VI) at an initial K_2CrO_4 concentration of 400 µg ml^{-1} (Fig. 2).

Cr (VI) reduction by bacterial supernatant

To check the effect of bacterial supernatant on chromium reduction, the

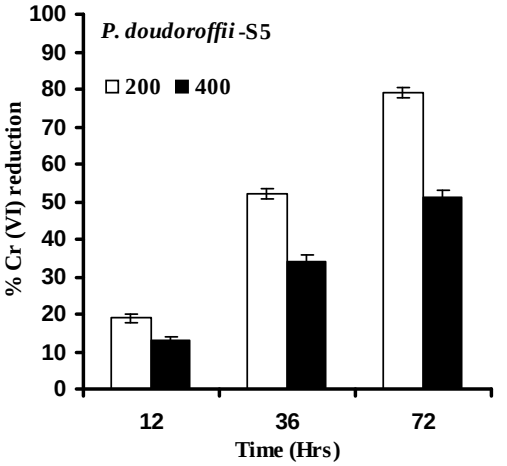

Fig. 2. Hexavalent chromium reduction potential of bacterial strains (*B. pumilus*-S4 and *P. doudoroffii*-S5) at different initial Cr (VI) concentration (200 and 400 µg/ml of K_2CrO_4) and incubation time (12, 36 and 72 h)

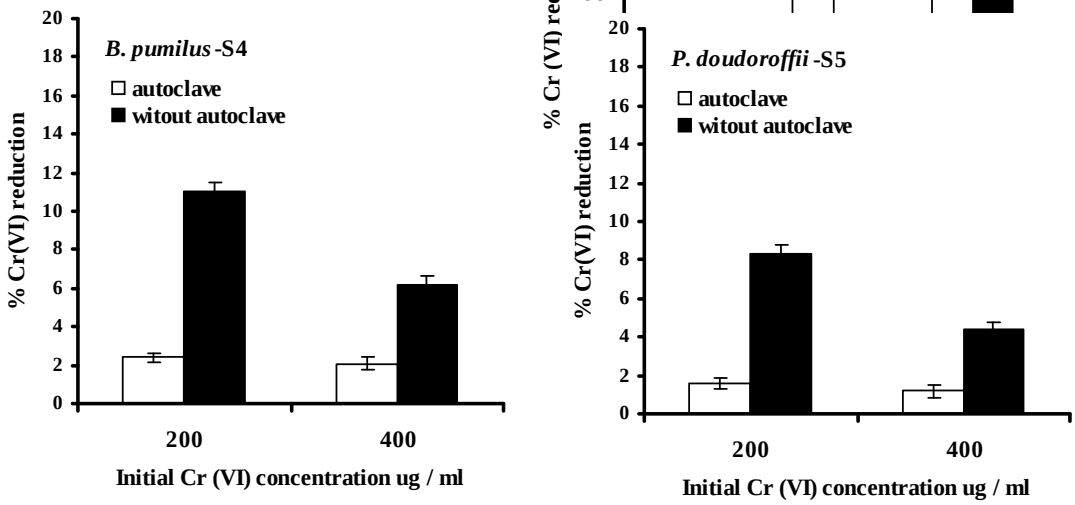

Fig. 3. Hexavalent chromium reduction potential of bacterial supernatant (*B. pumilus*-S4 and *P. doudoroffii*-S5) in the presence of 200 and 400 µg ml^{-1} of K_2CrO_4 at pH 7 and 37°C for 24 h incubation period. Bacterial supernatant was used i) as such ii) autoclaved at 121ºC for 15 min to denature the protein

supernatant from 24 h old cultures were obtained and utilized both as such and after autoclaving (to denature the protein involved in Cr (VI) reduction). Two different initial Cr (VI) concentrations (200 and 400 µg ml^{-1}) were used in this experiment. Un-autoclaved supernatant of strain *B. pumilus*-S4 was able to reduced 11% of Cr (VI) supplied after 24 h at an initial K_2CrO_4 concentration of 200 µg ml^{-1} (Fig. 3). While in the same time interval, autoclaved supernatant of this strain reduced 2.4% of the 200 µg ml^{-1} of K_2CrO_4 which is much less compared to un-autoclaved supernatant.

Effect of hydrophytes on Cr removal

After two weeks of incubation, in the presence of *B. pumilus*-S4, *Eichornia crassipes* accumulated 24% alone and 47% in bacterial presence at an initial K_2CrO_4 concentration of 50 µg ml^{-1} (Fig. 4). In the same time period and metal concentration bacterial strain *B. pumilus*-S4 removes 38% of Cr alone. While in the same time period, *Pistia stratiotes* removes 43% of Cr in the presence of *B. pumilus*-S4.

Almost same trends were seen in case of *P. doudoroffii*-S5 where strain alone removes 32% while in the presence of *Eichornia crassipes* and *Pistia stratiotes* removes 44% and 37% of Cr, respectively, at an initial K_2CrO_4 concentration of 50 µg ml^{-1} after two weeks (Fig. 4).

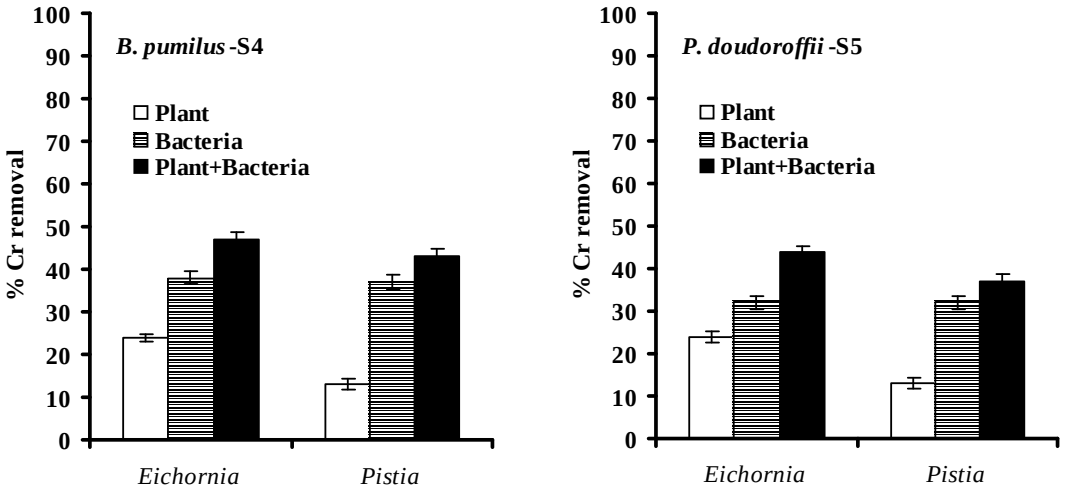

Fig. 4. Chromium removal potential of bacterial strains (*B. pumilus*-S4 and *P. doudoroffii*-S5) in the presence of *Eichornia crassipes* and *Pistia stratiotes* at an initial K_2CrO_4 concentration of 50 µg ml^{-1} for 15 days at 37°C. Plants and bacterial strain were used alone and in combinations

DISCUSSION

Increased pollution with an alarming rate not only affects our daily life but also changed the quality of ecosystem[21]. Hazardous chemicals released from industries are directly thrown in their wastewaters which get their way into fresh water[22]. Basically the present work deals with the detoxification/removal of carcinogenic Cr (VI) both by bacteria (*B. pumilus*-S4 and *P. doudoroffii*-S5) alone and in combination with free floating hydrophytes (*Eichornia crassipes* and *Pistia stratiotes*). Both these strain could resist Cr (VI) both on acetate minimal (2.2 mg ml^{-1}) and rich media (up to 20 mg ml^{-1}). These strains also show multiple heavy metal and antibiotic resistances. This resistance is a positive point because it will help the strain for their survival in the waste water treatment plant when ever they will be used for bioremediation purpose.

The present Cr accumulation study revealed that the accumulation is metabolically energy dependent mechanism. In the first 30 min the amount of Cr accumulated in the heat killed and living cells was not much different. But with the passage of time the heat killed cells become saturated and no further accumulation occurred. Also some Cr accumulation/deposition apartment

within the cell is also deformed to the heat. But in living cells the Cr accumulation was observed even after 720 min. Pawel *et al*[23] also observed that in yeast cells the amount of Cr accumulation was occurred in two phase, a rapid accumulation phase was followed by a slow time dependent increase. Ozdemir *et al*[24] while working on *Pantoea* sp. TEM18 showed that the metal adsorption increased during the first 15 min and after that its goes in to an equilibrium period.

Both these strains reduced Cr (VI) aerobically using citrate as one of the efficient electron donor. The %age of chromate reduction increased with increased in time period. *P. doudoroffii*-S5 reduced 14.2 mg Cr (VI) 36 h^{-1} at an initial concentration of 200 µg K_2CrO_4 ml^{-1}. It was observed that at 400 µg K_2CrO_4, the %age of Cr (VI) reduced was less but overall more amount of Cr (VI) was reduced in both strains. Verma *et al*[25] observed that strain *Bacillus brevis* isolated from tannery effluent could reduced 75.8% of Cr (VI) at an initial concentration of 180 µg Cr ml^{-1} within 28 h of incubation. Bacteria can reduce Cr (VI) both by soluble protein fractions and membrane fractions. In present case we have also checked the Cr reduction ability of soluble protein present in the supernatant and this activity was significantly decreased by de-nature these proteins. It means in both theses strains (*B. Pumilus-S4* and *P. doudoroffii*-S5) a well developed chromate reduction system associated with soluble protein exists. Beside microorganisms, certain plants are known to accumulate heavy metals, trace elements, organic or radioactive compounds in soils, groundwater, and industrial waste from their environment[26]. The wetland plants including free floating hydrophytes gained much attention because of their rhizofiltration and phytofiltration of toxic metals[27]. In the present work it was observed that the amount of Cr removal by bacterial strains was accelerated in the presence of hydrophytes (*Pistia stratiotes* and *Eichhornia crassipes*). Besides this they will support each other growth. Because the plants release carbon and energy source in the form of root exudates which help the bacteria strains. In turn these bacterial strains produce and liberate phytohormones which support hydrophytes growth. Becerra-Castro *et al*[28] also study the interaction of bacterial strains and plants to solubilise Ni in the soil and potentially improve phytoextraction strategies.

CONCLUSIONS

These results suggest that both these strains efficiently uptake and reduce toxic Cr (VI) from the solution and this process is augmented in the presence of *Pistia stratiotes* and *Eichornia crassipes*. So the present bioremediation method is a promising step towards the treatment of industrial waste water through the use of bacterial strains along with metal accumulator hydrophytes.

ACKNOWLEDGMENTS

Higher Education Commission, Pakistan is greatly acknowledge for the financial support for this work.

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