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EXPERIMENTAL ARTICLES

Arsenic and Chromium Reduction in Co-Cultures of Bacteria Isolated from Industrial Sites in Pakistan[1]

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Abstract—As(V)- and Cr(VI)-resistant bacteria were isolated from the industrial city Kasur, Pakistan. The 16S rRNA gene sequencing revealed that the highly resistant bacteria KS2-1, KS2-2, MWM81, and KSKE41 were related to *Bacillus* sp., *Rhodococcus* sp., *Cellulosimicrobium* sp., and *Exiguobacterium* sp., respectively. KS2-1 reduced As(V) up to 94% and MWM81 reduced Cr(VI) up to 45%. Co-cultures of KS2-1 and KS2-2 reduced As(V) up to 98%, whereas co-cultures of MWM81 and KSKE41 reduced Cr(VI) up to 55%. Bacteria living in same niches could work together to degrade contaminants which were common toxicants for them.

Keywords: chromium, arsenic, PICT, *Bacillus*, *Rhodococcus*, *Cellulomicrobium*, *Exguobacterium*

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Despite of its role in modernization of human societies, industrialization also has harmful effects on the environment and the biota, particularly due to the release of toxic substances such as metals. Arsenic (As) is a toxic metalloid which is being released into the environment in a variety of ways such as coal combustion, production of pigments for paints and dyes, smelting, mining, and tanning [1]. These anthropogenic influx results in increased concentrations of arsenic in groundwater. Drinking arsenic-contaminated water over long periods of time results in arsenicosis, which may cause patches on skin, cancer of skin, kidney, bladder, and lungs, as well as diseases of blood vessels [2]. Chromium (Cr) is also a toxic heavy metal released into the environment by various industrial processes such as leather tanning, electroplating, alloy preparation, metal finishing, and paint industry [3, 4]. In the environment, chromium is most frequently found as Cr(III) and Cr(VI). Cr(III) is less toxic and mobile, while Cr(VI) is a highly soluble, strong oxidizing agent [5]. Cr(VI) is reduced intracellularly to Cr(V), which reacts with nucleic acids and other cell components causing hazardous (mutagenic and carcinogenic) effects [6]. Arsenic- and chromium-contaminated soils harbor microorganisms that not only resist but also detoxify such metals. Biotransformation of chromium and arsenic by bacteria offers an inexpensive, environment-friendly substitute for chromium and arsenic remediation [7]. In this study we isolated bacteria from Kasur, Pakistan. Kasur is one of the oldest industrial cities of Pakistan and has had a large number of tanneries for decades. Most of the industries and tanneries in this part of the country release their wastes into the environment, which has resulted in elevated concentrations of arsenic, chromium, and other metals in the soil.

The goal of the present work was to isolate As(V)- and Cr(VI)-resistant bacteria from Kasur soils and to assess their capacity for As(V) and Cr(VI) reduction individually and in co-cultures.

MATERIALS AND METHODS

Isolation. Soil samples were collected from industrially contaminated sites of Kasur, Pakistan, and pH and temperature were recorded. Serial dilutions of these samples were plated on nutrient agar plates (LB media, pH 7.0) and incubated at 37°C for 24 h. Diverse bacterial colonies obtained were purified and transferred to nutrient media containing 1 mM Cr(VI) (K_2CrO_4) or 1 mM As(V) ($Na_2HAsO_4 \cdot 7H_2O$). The plates were incubated at 37°C for 24 h. Bacteria that survived this metal stress were subjected to higher concentrations of As(V) and Cr(VI). All experiments were performed in triplicates.

Determination of Minimal Inhibitory Concentration (MIC). Bacterial isolates were cultured in nutrient media (LB media, pH 7.0) containing As(V) or Cr(VI) in different concentrations: 10, 25, 50, 75, 100, 125, 150, 175, 200, 250 and 300 mM. Overnight cultures (50 μL) were used as inocula, and the media were incubated under agitation (200 rpm) for at least 72 h at 37°C. After incubation, the isolates that were found to have the highest minimal inhibitory concentrations (MIC) of these metals were selected. MIC of As(V) and Cr(VI) were also determined on minimal medium

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[8] and acetate minimal medium, [9] respectively. Two As(V)-resistant bacteria: KS2-1 and KS2-2, and two Cr(VI)-resistant bacteria: MWM81 and KSKE41 were selected for further studies.

Characterization and identification. Selected bacteria were characterized morphologically and biochemically. Genomic DNA was isolated using the Invitrogen Easy DNA kit. Polymerase chain reaction (PCR) amplification of the 16S rRNA genes was carried out using the universal primers 8F (AGAGTTTGATCCTTGGCTCAG) and 1492R (GCYTACCTTGTTACGACTT). Amplicons were purified using the Epoch GenCatch Advanced Gel Extraction Kit and sequenced at Macrogen, Korea. The presence of any chimera sequences was determined with Pintail [10].

Reduction of As(V) and Cr(VI). Selected bacteria were analyzed for their ability to reduce As(V) and Cr(VI). For Cr(VI) reduction assay, DeLeo and Ehrlich [11] medium supplemented with 10 mM Cr(VI) was used, whereas the basal mineral medium [12] supplemented with 20 mM As(V) was used for As(V) reduction assay. The cultures were incubated for 48 h along with the control flasks containing uninoculated media with As(V) or Cr(VI). After incubation, samples were taken out of the cultures, centrifuged and analyzed. Concentration of Cr(VI) reduced was determined reaction with S-diphenyl-carbazide (DPC) in the presence of phosphoric acid [13]. Spectrophotometry measurements were made at 540 nm. Concentration of As(V) reduced was determined by the method reported by Cummings, Caccavo, Fendorf, and Rosenzweig [14] and absorbance was recorded at 865 nm using a Thermo Scientific GENESYS 20 spectrophotometer.

Optimal pH and temperature for As(V) and Cr(VI) reduction. As(V) and Cr(VI) reduction was analyzed at different pH and temperatures. Bacterial isolates were inoculated into the media as described earlier and incubated at 28, 37 and 45°C to determine the optimal temperatures for As(V) and Cr(VI) reduction. To determine the pH optima, the cultures were inoculated into the media with As(V) or Cr(VI) with initial pH values of 3, 5, 7, and 9. The uninoculated controls were also set up to evaluate the abiotic effects of these parameters on As(V) and Cr(VI) reduction.

Effects of antibiotics and heavy metals on As(V) and Cr(VI) reduction. The ability of the isolates to reduce As(V) and Cr(VI) was also analyzed in the presence of antibiotics and heavy metals. Media containing Cr(VI) and As(V) were supplemented with 10 μg penicillin or 30 μg chloramphenicol, and inoculated as described earlier. Reduction of As(V) by KS2-1 and KS2-2 (As(V)-resistant bacteria) was also determined in the presence of 5 mM Cr(VI), and reduction of Cr(VI) by MWM81 and KSKE41 (Cr(VI)-resistant bacteria) was also determined in the presence of 5 mM As(V). Reduction of these metals was also analyzed in the presence of 5 mM manganese ($MnSO_4$). Controls were set up to evaluate the abiotic effects of penicillin, chloramphenicol, and the metals on As(V) and Cr(VI) reduction.

Table 1. Combination of co-cultures

Metal	Bacteria in co-cultures
As(V)	KS2-1 + MWM81
	KS2-1 + KSKE41
	KS2-2 + MWM81
	KS2-2 + MWM81
	KS2-1 + KS2-2
Cr(VI)	MWM81 + KS2-1
	MWM81 + KS2-2
	KSKE41 + KS2-1
	KSKE41 + KS2-2
	MWM81 + KSKE41

Co-cultures. The bacteria were co-cultured to check whether they can coexist and enhance the reduction of Cr(VI) and As(V) in co-cultures. Overnight cultures (100 μL) were inoculated in the media containing 20 mM As(V) and in media containing 10 mM Cr(VI) in combinations as shown in Table 1. The cultures were incubated under shaking (200 rpm) for 48 h at 37°C. After incubation, 50 μL of these cultures were plated on nutrient agar (LB) and incubated for 24 h at 37°C for CFU (colony forming units) count. Samples were taken out for As(V) and Cr(VI) determination as mentioned earlier.

Amplification of arsB and ACR3 genes. PCR amplification of the genes responsible for As(V) resistance, *arsB* and *ACR3*, was performed. For *arsB* amplification, darsB1F: GGTGTGGAACATCGTCTGGAAYGCNAC and darsB1R: CAGGCCGTACACCACCAGRTACATNCC primers were used. For *ACR3* amplification, dacr5F: TGATCTGGGTCATGATCTTCCCVATGMTGVT, and dac4R: CGGCCACGGCCAGYTCRAARAARTT [15] primers were used. PCR cycling conditions used were the same as reported by Achour, Bauda, and Billard [15].

RESULTS

Isolation. Soil samples collected were grayish brown in color, with pH 7.5 and the temperature of 39°C at the time of sampling. Forty different bacteria were obtained on nutrient media. The bacteria were purified and transferred to media supplemented with As(V) or Cr(VI), 1 mM each. Bacteria that survived the metal stress in As(V)- and Cr(VI)-supplemented media were selected for further studies.

Minimal Inhibitory Concentration (MIC). Bacteria showed varied patterns of resistance against As(V) and Cr(VI) on nutrient media as shown in Figs. 1 and 2, respectively. KS2-1 and KS2-2 had highest MIC of As(V) (up to 250 mM) (Fig. 1). However, in minimal

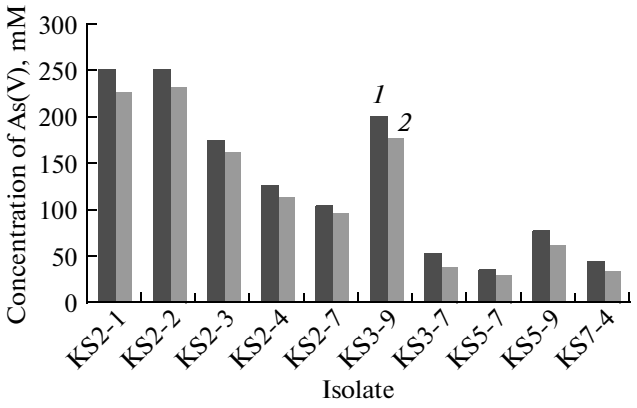

Fig. 1. Minimal inhibitory concentrations (MIC) of As(V) for As(V)-resistant bacteria in nutrient media (*1*) and minimal media (*2*). MIC of these bacteria was lower when cultured in minimal media.

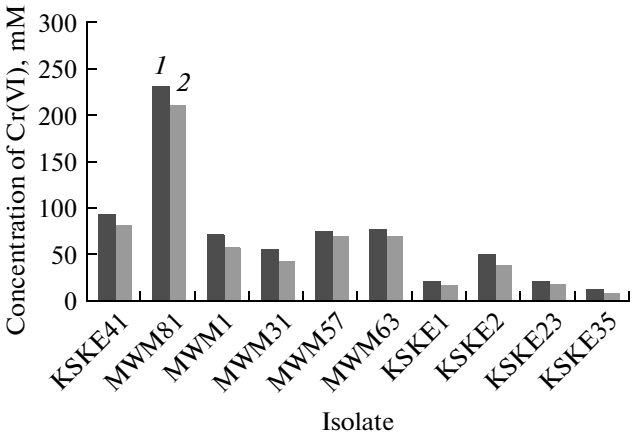

Fig. 2. Minimal inhibitory concentration (MIC) of Cr(VI) for Cr(VI)-resistant bacterial in nutrient media (*1*) and minimal media (*2*). MIC of these bacteria was lower when cultured in minimal media.

media its resistance against As(V) was lower. KS2-1 resisted 225 mM whereas KS2-2 resisted 230 mM of As(V) in minimal media (Fig. 1). For Cr(VI), the highest resistance was shown by MWM81 and KSKE41 (up to 250 and 100 mM, respectively) (Fig. 2). Resistance against Cr(VI) was also lower in minimal media. MWM81 and KSKE41 resisted Cr(VI) up to 230 and 85 mM on minimal media, respectively (Fig. 2). These four bacteria with higher resistance to As(V) and Cr(VI) were selected for further studies.

Characterization and identification. Selected bacteria were characterized and the tests were performed, some of which are listed in Table 2. BLAST results of the 16S rRNA gene sequences of KS2-1, KS2-2, MWM81, and KSKE41 revealed that these bacteria were closely related to *Bacillus* sp. (100%), *Rhodococcus* sp. (99%), *Cellulosimicrobium* sp. (99%) and *Exiguobacterium* sp. (99%), respectively. The sequences were submitted in the NCBI GenBank under accession numbers JX555882, JX535360, JX535362 and JX535361 for KS2-1, KS2-2, MWM81, and KSKE41, respectively.

Optimal pH and temperature for As(V) and Cr(VI) reduction. *Bacillus* sp. KS2-1 and *Rhodococcus* sp. KS2-2 reduced As(V) most efficiently at 37°C. As(V) reduction ability of these bacteria was the lowest at 45°C. Optimal pH for As(V) reduction was 7 for both of these bacteria (Fig. 3). Optimal temperature for Cr(VI) reduction by *Cellulosimicrobium* sp. MWM81 was 37°C, whereas fox *Exiguobacterium* sp. KSKE41 it was 28°C. Cr(VI) reduction by both of these bacteria was most efficient at pH 7, and reduction was most the lowest at pH 3 (Fig. 4).

Table 2. Major characteristics of selected isolates

Tests	KS2-1	KS2-2	MWM81	KSKE41
Gram stain	Positive	Positive	Positive	Positive
Motility	Motile	Non motile	Non motile	Motile
Catalase	Positive	Positive	Positive	Positive
Oxidase	Positive	Negative	Negative	Positive

Effects of antibiotics and heavy metals on As(V) and Cr(VI) reduction. As(V) and Cr(VI) reduction by these bacteria was affected in the presence of antibiotics (Figs. 3 and 4). *Bacillus* sp. KS2-1 reduced As(V) only up to 19 and 7% in the presence of penicillin and chloramphenicol, respectively. Similarly As(V) reduction by *Rhodococcus* sp. KS2-2 was also decreased and only up to 2 and 5% As(V) was reduced in the presence of penicillin and chloramphenicol, respectively (Fig. 3). Penicillin and chloramphenicol also affected Cr(VI) reduction by *Exiguobacterium* sp. KSKE41 and *Cellulosimicrobium* sp. MWM81. *Exiguobacterium* sp. KSKE41 reduced Cr(VI) only up to 15 and 20%, whereas *Cellulosimicrobium* sp. MWM81 reduced Cr(VI) only up to 14 and 11% in the presence of penicillin and chloramphenicol respectively (Fig. 4). As(V) reduction ability of KS2-1 and KS2-2 was also decreased in the presence of Cr(VI) and Mn(II). Likewise, Cr(VI) reduction by KSKE4 and MWM81 also decreased in the presence of As(V) and Mn(II).

As(V) and Cr(VI) reduction and co-cultures. *Bacillus* sp. KS2-1 showed highest As(V) reduction and reduced up to 95% of As(V), whereas *Rhodococcus* sp. KS2-2 reduced 14% of As(V). *Cellulosimicrobium* sp. MWM81 also reduced As(V) (~10%) (Fig. 5). The highest Cr(VI) reduction was shown by *Cellulosimicrobium* sp. MWM81 which reduced Cr(VI) up to 45%, whereas *Exiguobacterium* sp. KSKE41 reduced Cr(VI) up to 35% (Fig. 6).

In co-cultures in As(V)-supplemented media, the bacteria showed almost equal CFU counts. However, in co-cultures in Cr(VI)-amended media the percent-

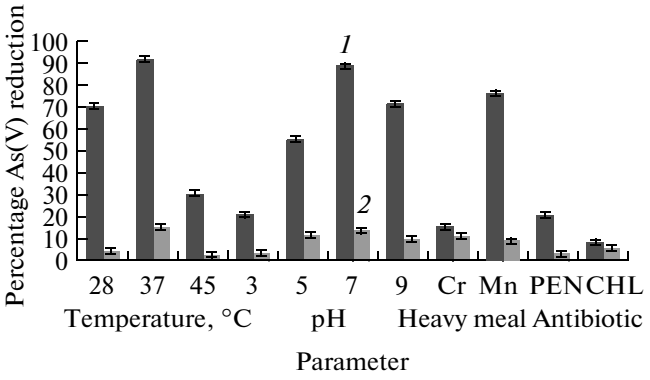

Fig. 3. Effect of temperature, pH, heavy metals and antibiotics on As(V) reduction potential of bacterial isolates *Bacillus* sp. KS2-1 (*1*) and *Rhodococcus* sp. KS2-2 (*2*).

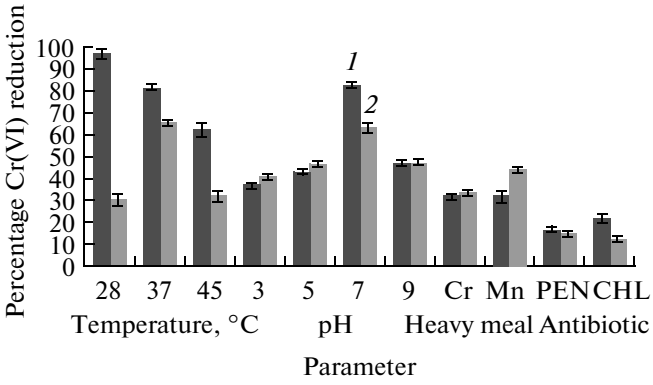

Fig. 4. Effect of temperature, pH, heavy metals and antibiotics on Cr(VI) reduction potential of bacterial isolates *Exguobacterium* sp. KSKE41 (*1*) and *Cellulosimicrobium* sp. MWM81 (*2*).

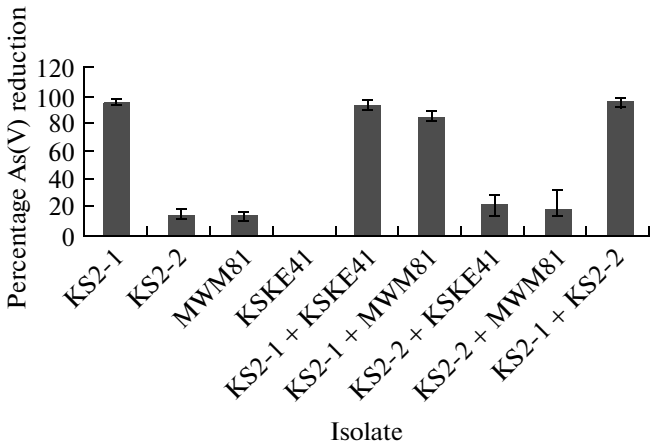

Fig. 5. Percentage of As(V) reduction by bacterial isolates in pure cultures and in co-cultures. As(V) reduction was enhanced in co-cultures of these bacteria.

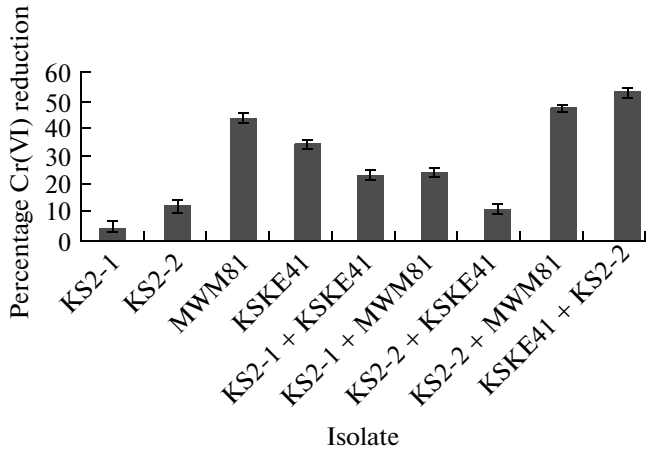

Fig. 6. Percentage of Cr(VI) reduction by bacterial isolates in pure cultures and in co-cultures. Cr(VI) reduction was enhanced in co-cultures of these bacteria.

age growth of co-cultured bacteria was different. *Exiguobacterium* sp. KSKE41 suppressed the growth of *Cellulosimicrobium* sp. MWM81 and about 80% of CFU/mL were of *Exiguobacterium* sp. KSKE41. In co-cultures of *Rhodococcus* sp. KS2-2 and *Exiguobacterium* sp. KSKE41, both suppressed each other's growth. Only 6 CFU/mL were obtained and all were of *Rhodococcus* sp. KS2-2. In co-cultures of *Rhodococcus* sp. KS2-2 and *Cellulosimicrobium* sp. MWM81, only 10% of the CFU were of *Rhodococcus* sp. KS2-2 and 90% were of *Cellulosimicrobium* sp. MWM81.

As(V) reduction potential of *Bacillus* sp. KS2-1 increased when it was co-cultured with *Rhodococcus* sp. KS2-2, resulting in reduction of up to 98% of As(V) (Fig. 5). As(V) reduction by KS2-2 was ~2 fold increased when co-cultured with MWM81 and KSKE41. Cr(VI) reduction potential of *Cellulosimicrobium* sp. MWM81 increased when cultured with *Exiguobacterium* sp. KSKE41 and *Rhodococcus* sp. KS2-2, resulting in reduction of up to 55 and 48% Cr(VI), respectively (Fig. 6).

Amplification *arsB* and *ACR3* genes. PCR amplification of *arsB* using the primers described earlier produced ~750 bp amplicons. No amplicon was found for the *ACR3* gene.

DISCUSSION

Kasur is a major industrial city of the province of Punjab, Pakistan. Most of the tanneries located in this city have been dumping their wastes in the surroundings for years. Microbial flora of this region is likely to develop pollution-induced community tolerance (PICT) to Cr(VI), As(V), and other toxic compounds used in the tanning industry [16, 17]. A large diversity of bacteria was isolated from soil samples of Kasur city on nutrient media. However, most of these bacteria were unable to survive As(V) and Cr(VI) stress when

they were transferred to media supplemented with these metals. Not all bacteria present in contaminated soils could tolerate the toxicants present in the soil. As soil is a complex matrix containing aggregates, micropores and microsites, the availability of heavy metals and other contaminants is not the same throughout the complex soil matrix. As a result, the ability of the microorganisms to resist metals varies depending on their location in the soil matrix [18]. The non-resistant bacteria isolated in this study probably have not been directly exposed to the toxic metals in the soil. As(V)- and Cr(VI)-resistant bacteria showed different MIC values in nutrient media and in minimal media. In complex nutrient media, metals can bind and chelate organic components of the medium. Consequently, total amount of the metal available to bacteria is not the same as the amount added to the medium [19]. Metals were more readily available to the bacteria in minimal media than in nutrient media. Therefore, MIC of the bacteria decreased in minimal media. The selected bacteria KS2-1, KS2-2, MWM81, and KSKE41 were identified as *Bacillus* sp., *Rhodococcus* sp., *Cellulosimicrobium* sp. and *Exiguobacterium* sp., respectively by 16S rRNA gene sequencing. Bacteria of these genera are known to reduce As(V) and Cr(VI) to varied concentrations [20–24]. As these bacteria were isolated from polluted environments where bacterial communities face other stresses as well, As(V) and Cr(VI) reduction potential was also determined in the presence of other metallic ions and antibiotics. Penicillin, chloramphenicol and manganese affected the growth of the bacteria. Consequently, As(V) and Cr(VI) reduction potential of these bacteria was impaired. Due to environmental pressure, these strains developed the strategies to combat a wide range of contaminants. Metals and antibiotics resistance can be co-selected and many cases of associations between metals resistance and antibiotics resistance in microorganisms have been reported [25]. Associations between antibiotics resistance and As(V) resistance [26], as well as associations between antibiotics resistance and Cr(VI) resistance [27] have also been reported in microorganisms, PCR amplification and sequencing of *arsB* gene showed that the high resistance of As(V) by these bacteria is due to the presence of a membrane transport pump for As(V) encoded by the *arsB* gene. The 45 kDa membrane protein ArsB of *Escherichia coli* has been extensively studied and consists of 12 transmembrane helices [28]. As(V) taken up by microbial cells is enzymatically reduced by arsenate reductase (arsC) and transported out of the cells by arsB [29]. In co-cultures of these bacteria, the increased reduction of As(V) correlated with the CFU counts which showed that the bacteria could co-exist and hence could function together to reduce As(V). In Cr(VI) co-cultures, although *Exiguobacterium* sp. KSKE41 suppressed the growth of *Cellulosimicrobium* sp. MWM81, Cr(VI) reduction was higher than in pure cultures. Reduction of different metals by microbial co-cultures has also been studied by many scientists. Co-cultures of two *Bacillus* species were used in order to achieve greater selenium reduction [30]. Heavy metals reduction by microbial co-cultures has also been reported along with biodegradation of toxic compounds [31, 32].

This study shows that a diversity of bacteria is present in industrially contaminated soils. Microbes that are exposed to contaminants develop the strategies to resist and detoxify these contaminants. Bacterial communities living in contaminated soils work together to detoxify the contaminants that are common toxicants for them. Co-cultivation of selected As(V)- and Cr(VI)-resistant bacteria revealed that the amount of As(V) and Cr(VI) reduced was higher than in pure cultures of each organism. A larger increase in reduction is expected if bacteria happen to be of the same genus (closely related) or known to coexist in various niches. Such co-cultures can prove powerful biotechnological tools to remediate contaminated soils as compared to remediation by pure cultures.

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