

Removal of Hexavalent Chromium *in Vitro* and from Contaminated Soils by Chromate-Resistant Fungi from Chromium Deposits

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Three chromate-resistant filamentous fungi, strains N2, N3 and N5, were selected from seven independent fungal isolates indigenous to Cr(VI) contaminated soil based on their ability to decrease hexavalent chromium level in the growth medium. Morphophysiological studies identified strain N2 as an *Aspergillus* sp. and strains N3 and N5 as *Penicillium* sp.. After 192 h growth in a medium containing 60 mg/L Cr(VI) and at near neutral pH, *Aspergillus* sp. N2 decreased the Cr(VI) concentration in the growth medium to a virtually undetected level, whereas both *Penicillium* sp. N3 and *Penicillium* sp. N5 decreased the Cr(VI) concentration by about 80%. However, acidic condition inhibited the Cr(VI) concentration reducing ability of these strains. Interestingly, Cu(II), as a coexisting ion, enhanced the Cr(VI) reducing ability of *Penicillium* sp. N3 at strong acid pH condition. In both slurry-phase and water-phase bioremediation assays, all three strains decreased the levels of Cr(VI) in polluted soil samples, suggesting that these chromate-resistant filamentous fungi might be useful in cleaning up chromium contaminated sites.

Key words: *Aspergillus* sp., bioremediation, chromate-resistant fungi, Cr(VI) removal, *Penicillium* sp., polluted soils

1. Introduction

Chromium (Cr), a widespread environmental pollutant, is released from various industries including tanneries, metal cleaning and processing, chromium plating, wood processing, and alloy formation^{3,30}. In the developing as well as in the underdeveloped countries, the industrial effluents are released directly or indirectly into the natural water resources, mostly without proper treatment, posing a major threat to the environment¹¹. Among the different oxidative forms of chromium, the hexavalent chromium (Cr(VI)) is the most toxic and carcinogenic due to its high solubility in water, rapid permeability through biological membranes, and subsequent interaction with the intracellular proteins and nucleic acids^{13,21,26}. Heavy metals in general cannot be biologically transformed into more or less toxic products and, hence, persist indefinitely in the environment. Although presence of Cr(VI) causes the chromate toxicity, further reduction leads to the formation of stable, less soluble, and less toxic Cr(III)⁴. Thus, reduction of potentially toxic Cr(VI) to less toxic Cr(III) is a useful process for remediation of Cr(VI) affected environments¹⁹.

Microbial viability is essential for biotransformation as these reactions are enzyme mediated. Generally metal ions are converted into insoluble form by specific enzyme medi-

ated reactions and are removed from the aqueous phase²². There are reports of using live microbial systems for the purpose of remediation of contaminated soils and waters⁷. Higher fungi, yeast, bacteria, seaweed and materials derived from plants (such as charcoal) are abundantly available in nature and can be useful sources for low cost biosorbents^{6,29}. The use of microbial cells as biosorbents for heavy metals is a potential alternative to conventional methods that are used to decontaminate liquid wastes. Several bacteria possess chromate reductase activity that can convert Cr(VI) to a much less toxic and less soluble Cr(III), and thus the reduction of Cr(VI) by these enzymes offer affordable means for chromate bioremediation¹². Detoxification of Cr by naturally occurring microorganisms therefore provides a viable option to protect the environment from chromium toxicity. Moreover, continuous exposure of microbial populations to heavy metal containing polluted environments selects resistant strains with higher level of tolerance to heavy metals¹⁸.

Some microorganisms are able to interact with different forms of Cr, making them attractive options for use in the field of environmental biotechnology. In this respect, it is noteworthy that the use of microbial biomass for the removal of Cr from the industrial wastewater and polluted water has already been recognized^{6,24}. Properties of some the micro-

organisms to both tolerate and reduce Cr(VI) enable their application in biotechnological processes focusing on detoxification of hexavalent Cr. Chromate resistance has been described in bacteria and fungi isolated from the Cr-contaminated environments. In the laboratory, chromate-resistant fungal strains were obtained by mutagenic induction⁶. Yeast strains isolated from the Cr-contaminated environments include those mostly from the genera *Candida*^{2,23} and to a minor extent from the genera *Rhodospiridium*²³. In these yeast strains, the general mechanism of chromate resistance is limited to the increased ion uptake rather than the chemical reduction of the toxic specie^{2,23}. However, other yeasts such as *Candida utilis*²⁰ and *Candida maltosa*²⁵ showed partial ability to reduce Cr(VI) and also the capability to accumulate Cr in the biomass. There are also several recent reports examining the uptake and accumulation of Cr(III) and (or) Cr(VI) by different yeasts^{5,14} and by filamentous fungi^{8,31}.

In this work, we have isolated two chromate-resistant strains of filamentous fungi indigenous to chromium deposit, characterized their abilities to remove Cr(VI) from the contaminated soil.

2. Materials and Methods

2.1. Isolation of fungal strains and chromate resistance test

Chromate-resistant filamentous fungi were isolated from chromium deposits in Fukuoka, Japan. A sample suspension was prepared for each sample by adding about 1 g of the fungi containing chromium deposit to 10 mL physiological saline solution. One hundred microliter of each sample suspension was dropped into a culture plate, PG agar media containing 500 mg/L Cr(VI) was poured on the top, and the agar plates were incubated at 30°C for 1 week. The microbial colonies (fungi) appearing on the culture plates were isolated, purified and characterized based on their morphological structures such as color, texture, and diameter of the mycelia, and microscopic observation of the spore formation. Seven strains (N2, N3, N4, N5, N6, N7, N8) were isolated from the PG solid medium containing 500 mg/L of Cr(VI), and further purified on culture plates. Finally, the fungi were inoculated from the plates onto the agar slants and stored at 4°C until needed for further experiments. The PG agar medium consisted of 5 g bacto-peptone, 2 g glucose, 15 g agar in 1.0 L deionized water. The pH value of the medium was adjusted to 6.0 with 6 M NaOH and 6 M HCl. Chromate-resistance test of the isolated strains was performed using PG liquid medium containing different concentrations of chromium oxide (0, 50, 100, 500 and 1000 mg/L) as Cr(VI).

2.2. Cr(VI) removal assay in liquid media

Each isolated strain was suspended in sterilized water and the spore concentration was adjusted to approximately 1×10^7 spores/mL. One milliliter of this spore suspension was

used for inoculating one hundred milliliter of a Cr(VI) containing liquid test medium in a 300 mL conical flask and incubated at 30°C for 8 days on a 60 rpm shaking incubator. The liquid test medium contained the same ingredients as the PG agar medium described above except agar. After autoclaving the medium at 120°C for 20 min, appropriate amount of chromium oxide solution was aseptically added to the medium to prepare Cr(VI) containing liquid test medium of various final concentrations (25, 60, 100 and 200 mg/L).

The influence of pH on Cr(VI) removal was examined by varying the pH of the reaction mixture from 3 to 6 and using the initial Cr(VI) concentration of 60 mg/L.

Stock solutions of copper sulfate and zinc sulfate as Zn(II) were prepared in distilled water, filter sterilized and appropriately diluted (50 mg/L final) in the PY liquid medium containing an initial Cr(VI) concentration of 60 mg/L for determining the influence of Cu(II) or Zn(II) on Cr(VI) removal.

2.3. Cr(VI) removal assay in contaminated soils

Contaminated soil samples were collected from the chromium contaminated sites at Fukuoka, Japan. Soil samples were manually collected and transported to the laboratory in airtight polythene containers. The pH of the soil was 6.5.

Two types of laboratory-scale bioremediation assays were carried out. In one type of assay (slurry-phase bioremediation assay), the mycelial biomass, obtained from a culture grown for 3 days in liquid medium as described in the previous section, was transferred to 50 mL of fresh PG liquid medium, followed by the addition of 10 g of contaminated soil. At this point, the DMW (Dry Mycelium Weight) of each fungi was about 0.5 g/L. At various times during the course of incubation, aliquots were removed, centrifuged at $4200 \times g$ for 20 min and the supernatant was used for the determination of Cr(VI) concentration (see below).

In the second assay (water-phase bioremediation assay), 10 g of contaminated soil was washed by incubating with 90 mL of distilled water for 24 hours on a 100 rpm shaking incubator. The pH value of the distilled water was adjusted to 8.0 with 6 M NaOH and 6 M HCl. Mycelial biomass, obtained from a culture grown for 3 days in liquid medium as described in the previous section, was transferred to 10 mL of fresh PG liquid medium, followed by the addition of 90 mL of the wash supernatant. At this point, the DMW of each fungi was about 0.5 g/L. At various times during the course of incubation, aliquots were removed, centrifuged at $4200 \times g$ for 20 min and the supernatant was used for the determination of Cr(VI) concentration (see below).

2.4. Growth assessment of microorganisms

Dry mycelium weight (DMW) as a growth measure was determined by drying the cells from the culture at 105°C for 24 h in an oven. DMW was defined as mycelium weight per 100 mL of liquid culture medium.

2.5. Measurement of Cr (VI) and total Cr concentration

Concentrations of Cr(VI) were determined by the diphenylcarbazide method¹⁶, and total Cr was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES, PerkinElmer Japan Co., Ltd.). Mycelia were removed from the fermentation broth by filtration through filter paper and centrifugation at $4200 \times g$ for 20 min. The supernatant fluid was filtered through a 0.45- μm -pore-size membrane filter (Toyo Roshi Kaisha, Ltd.). Five mL of culture supernatant was aseptically removed and transferred to a glass test tube. The sample was acidified with 1 mL of 10% H_2SO_4 . The sample was then treated with 1 mL of a 10 mg/mL diphenylcarbazide solution in acetone, and distilled water was added to a total volume of 50 mL. Cr(VI) reacts with diphenylcarbazide to form a bright pink color, the intensity of which is directly proportional to the Cr(VI) concentration. This solution was shaken for 30 s, and after 5 min the absorbance at 540 nm was read on a UV-vis spectrophotometer (Amersham Pharmacia Biotech Co., Ltd.). In addition, this solution was analyzed by ICP-AES. Cr(VI) concentrations were estimated from standard curves generated with known amounts of Cr(VI) using potassium chromate in the growth medium.

3. Results

Seven independent chromate-resistant filamentous fungi were isolated from the chromium deposits, which are labeled here as N2, N3, N4, N5, N6, N7 and N8. All seven strains were tested for their ability to decrease the Cr(VI) concentration in the growth media at near neutral (pH 6.0) and strong acidic (pH 3.0) conditions. As shown in Fig. 1, all seven fungal strains were able to efficiently decrease the Cr(VI) concentration in the growth medium; however, there were obvious differences in their efficiency of decreasing the Cr(VI) concentration. In the case of strong acid pH condition (pH 3.0), the Cr(VI) concentration in the N2 culture medium dropped from its initial concentration of 100 mg/L to 30 mg/L (~70% reduction) after 192 h incubation. During the same time period and similar strong acidic pH condition, the strains N3, N5 and N7 decreased the Cr(VI) concentration in the growth media by 60%, and the strains N4, N6 and N8 decreased the Cr(VI) concentration in the growth media by 35%, 50% and 45%, respectively. On the other hand, at the near neutral pH condition, the N5 and N6 strains decreased the Cr(VI) concentration by approximately 60%, the N2, N3 and N8 strains decreased the Cr(VI) concentration by about 50%, and the strains N7 and N4 decreased the Cr(VI) concentration by about 40% and 30%, respectively. The fungal isolate N2 was identified as *Aspergillus* sp., whereas the isolates N3 and N5 were identified as *Penicillium* sp.¹⁵; however, we do not know the identity of rest of the four isolated fungal strains. Thus, we used *Aspergillus* sp. N2, *Penicillium* sp. N3, and *Penicillium* sp. N5 for further investigation. All three strains (N2,

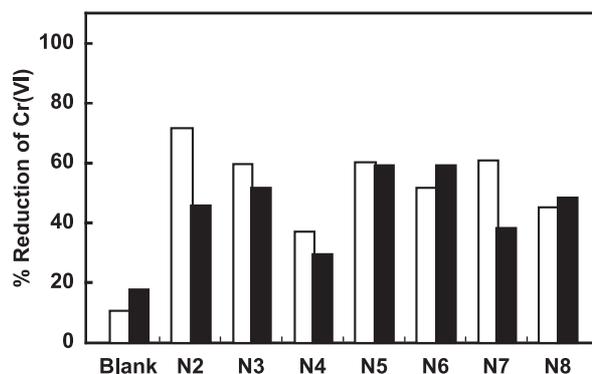


Fig. 1. Cr(VI) remaining (%) in the growth medium of cultures of seven (N2, N3, N4, N5, N6, N7 and N8) chromate-resistant fungal strains isolated from the chromium deposits. Each fungal strain was grown in the PG medium at 30°C for 192 h. The initial concentration of Cr(VI) in the medium was 100 mg/L. White bars depict percentage of Cr(VI) remaining after 192 h growth in the medium at near neutral pH condition (pH 6.0). Black bars depict percentage of Cr(VI) remaining after 192 h growth in the medium at an acidic condition (pH 3.0).

N3 and N5) showed significant growth in the presence of up to 25 mg/L Cr(VI), and were able to grow, albeit very slowly, even in the presence of 1000 mg/L Cr(VI).

Next, we compared the abilities of the *Aspergillus* sp. N2, *Penicillium* sp. N3, and *Penicillium* sp. N5 strains in lowering the concentration of Cr(VI) (initial concentration 60 mg/L) in the culture medium. Fig. 2A shows the results when the medium pH was near neutral (pH 6.0). After 192 h of incubation in medium, *Aspergillus* sp. N2 exhibited a remarkable efficiency to diminish the Cr(VI) level in the growth medium. The other two strains, N3 and N5, were also able to decrease the Cr(VI) concentration in the growth medium by about 80%. In the *Aspergillus* sp. N2 culture, the yellow color due to the soluble Cr(VI) turned into slightly turbid brownish color (result not shown). In contrast, when grown in strongly acidic medium (pH 3.0), these strains were able to decrease the Cr(VI) concentration in the growth medium to a significantly lower extent than the neutral pH condition (Fig. 2B). Whereas *Aspergillus* sp. N2 and *Penicillium* sp. N3 decreased the Cr(VI) concentration in the medium under the strong acidic condition by 50%, *Penicillium* sp. N5 was able to reduce the Cr(VI) concentration by only 30%. However, the Cr(VI) concentration also decreased by about 30% in the medium of the control culture without any inoculum.

Next, we cultured *Aspergillus* sp. N2, *Penicillium* sp. N3, and *Penicillium* sp. N5 in liquid media containing different concentrations (25–200 mg/L) of Cr(VI) and examined how effectively they could remove chromate from the medium after 192 h growth. Results of these experiments are shown in Fig. 3. As shown, the initial concentration of Cr(VI) in the culture medium clearly affected the ability of these strains to decrease the Cr(VI) concentration in the medium after 192 h of growth, and all three strains showed very

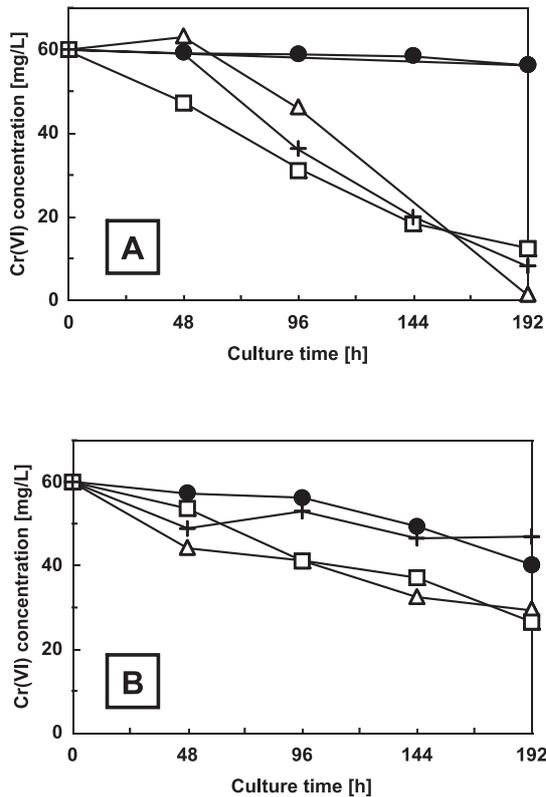


Fig. 2. Time course of decrease in Cr(VI) concentration (mg/l) in the growth medium of cultures initiated in PG medium at (A) near neutral pH 6.0 (B) acidic pH 3.0 supplemented with 60 mg/L of Cr(VI).

Symbols represent: —●—, blank; —△—, *Aspergillus* sp. N2; —□—, *Penicillium* sp. N3; and —+—, *Penicillium* sp. N5.

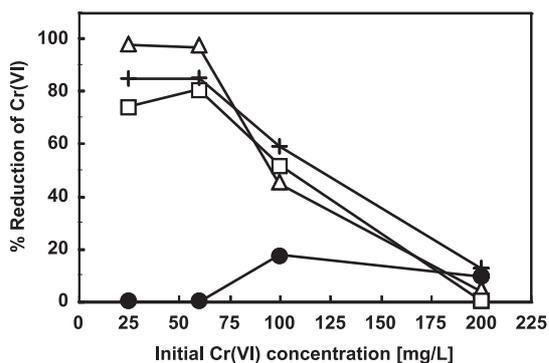


Fig. 3. Percent decrease in Cr(VI) concentration in the growth medium containing different concentrations of Cr(VI).

Each fungal strain was grown in the PG medium at 30°C for 192 h. Symbols represent: —●—, blank; —△—, *Aspergillus* sp. N2; —□—, *Penicillium* sp. N3; and —+—, *Penicillium* sp. N5.

similar efficiency pattern in removing Cr(VI) from the medium. At lower initial Cr(VI) concentrations (25 mg/L and 60 mg/L), *Aspergillus* sp. N2 completely removed the Cr(VI) from the medium; in contrast, at initial Cr(VI) concentrations of 100 mg/L and 200 mg/L, N2 decreased the Cr(VI) concentration in the media by 45% and 5%, respec-

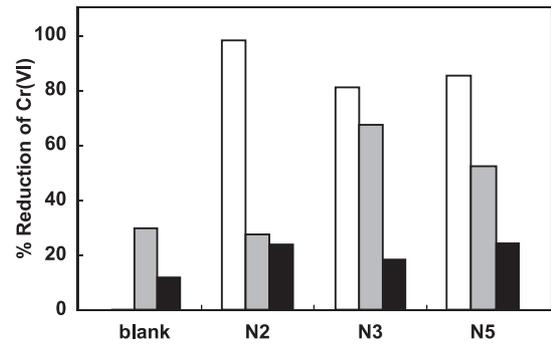


Fig. 4. Effect of divalent cations on the Cr(VI) reducing properties of the isolated fungi. Each strain was grown in the PG medium at 30°C for 192 h. White bars, % reduction of Cr(VI) concentration when the strains were grown in the medium containing 60 mg/L Cr(VI). Gray bars, % reduction in the Cr(VI) concentration when the strains were grown in the medium containing 60 mg/L Cr(VI) plus 50 mg/L Cu(II). Black bars, % reduction in the Cr(VI) concentration when the strains were grown in the medium containing 60 mg/L Cr(VI) plus 50 mg/L Zn(II).

tively. Similarly, at initial Cr(VI) concentrations of 25 mg/L, 60 mg/L and 100 mg/L, *Penicillium* sp. N3 decreased the Cr(VI) concentration in the growth medium by 75%, 75% and 60%, respectively, but failed to decrease the Cr(VI) concentration when the initial concentration of Cr(VI) in the medium was 200 mg/L. Likewise, at initial Cr(VI) concentrations of 25 mg/L, 60 mg/L, 100 mg/L and 200 mg/L, *Penicillium* sp. N5 decreased the Cr(VI) concentration in the growth medium by 85%, 85%, 60% and 20%, respectively. Thus, the higher initial Cr(VI) concentration was, the lower rate of Cr(VI) removal.

Microbial growth and Cr(VI) removal are influenced by many environmental factors¹⁶, one of which is be the concentration of coexisting metal ions. It is known that copper and zinc commonly present in the Cr(VI) contaminated wastewater or Cr(VI) contaminated soil^{23,34}. Thus, we next investigated the influence of the coexisting Cu(II) and Zn(II) ions on the Cr(VI) removing properties of *Aspergillus* sp. N2, *Penicillium* sp. N3 and *Penicillium* sp. N5, and the results are shown in Fig. 4. These experiments were performed using the culture medium containing 60 mg/L of Cr(VI) as the base growth medium to which either Cu(II) or Zn(II) was added to a final concentration of 50 mg/L. As a coexisting ion, both Cu(II) and Zn(II) diminished the ability of each strain in decreasing the Cr(VI) concentration in the growth medium, and the diminishing effect of Zn(II) was higher than Cu(II). Diminishing effect of Cu(II) was, however, influenced by the initial pH of the growth medium. Fig. 5A shows the time course of decrease in Cr(VI) concentration in the presence of Cu(II) at neutral pH. Cu(II) affected the abilities of *Aspergillus* sp. N2, *Penicillium* sp. N3 and *Penicillium* sp. N5 in decreasing the Cr(VI) concentration. Although acidic condition inhibited the Cr(VI) concentration removing abilities of these strains, presence of trace amount of Cu(II) under acid condition actually

Table 1. DMW of isolated fungi that were grown in the presence of 60 mg/L of Cr(VI) at neutral pH (6.0)

Strain	Cr(VI)			Cr(VI) + Cu(II)		
	DMW [g/L]		% Reduction of Cr(VI) ^a	DMW [g/L]		% Reduction of Cr(VI) ^a
	48 h	192 h		48 h	192 h	
N2	0.00	0.44	97.3	0.00	0.01	26.8
N3	0.05	0.28	80.3	0.07	0.37	68.5
N5	0.00	0.40	84.9	0.00	0.34	55.4

^a Percentage reduction of Cr(VI) was evaluated from the Cr(VI) content remaining in the growth medium after 192 h growth

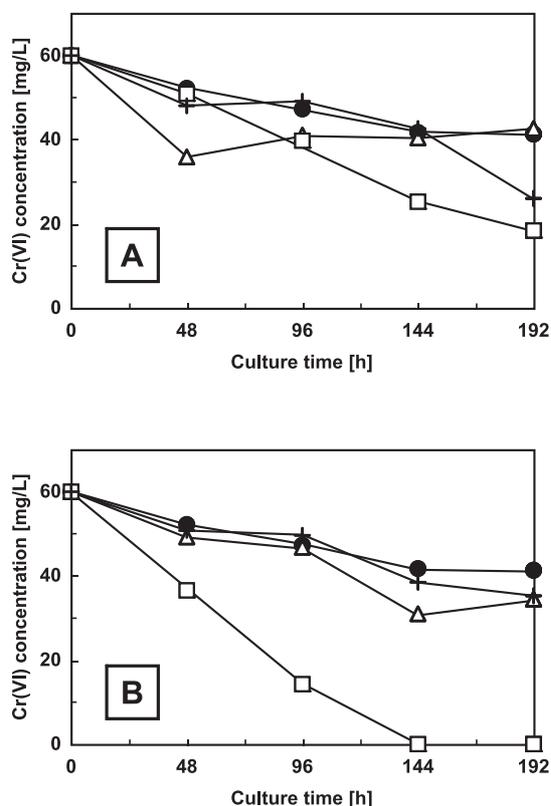


Fig. 5. Time course of decrease in Cr(VI) concentration in the growth media of cultures initiated in the PG medium supplemented with 60 mg/L of Cr(VI) and 50 mg/L of Cu(II) at (A) near neutral condition (pH 6.0) and (B) acidic condition (pH 3.0). Symbols represent: —●—, blank; —△—, *Aspergillus* sp. N2; —□—, *Penicillium* sp. N3; and —+—, *Penicillium* sp. N5.

enhanced the ability of *Penicillium* sp. N3, but not those of *Aspergillus* sp. N2 and *Penicillium* sp. N5, in decreasing the Cr(VI) concentration in the growth medium (Fig. 5B). Thus, when grown in the acidic medium (pH 3.0) containing 50 mg/L Cu(II), *Penicillium* sp. N3 decreased the Cr(VI) concentration from its initial concentration of 60 mg/L to almost undetected level after 144 h incubation.

Table 1 summarizes the DMW of N2, N3 and N5 at 48 and 192 h of incubation in growth medium at neutral pH containing different metal ions. In medium containing only Cr(VI), the DMWs of *Penicillium* sp. N3 after 48 h and 192 h incubation were 0.05 g/L and 0.28 g/L, respectively. Although *Aspergillus* sp. N2 and *Penicillium* sp. N5 did

not show any growth at 48 h incubation in the same medium, their DMWs after 192 h incubation (0.44 g/L and 0.40 g/L, respectively) suggested that they grew to the same extent. Under this growth condition, *Aspergillus* sp. N2, *Penicillium* sp. N3 and *Penicillium* sp. N5 decreased Cr(VI) concentration by 97%, 80% and 85%, respectively. The amount of Cr(VI) removed by the biomass of *Aspergillus* sp. N2, *Penicillium* sp. N3 and *Penicillium* sp. N5 was 111 mg/g, 143 mg/g, and 106 mg/g, respectively. In medium containing both Cr(VI) and Cu(II), the DMW of both *Penicillium* sp. N3 and *Penicillium* sp. N5 were approximately 0.35 g/L after 192 h incubation, whereas *Aspergillus* sp. N2 did not grow. Under this condition *Aspergillus* sp. N2, *Penicillium* sp. N3 and *Penicillium* sp. N5 decreased Cr(VI) concentration in the growth media by 23.9%, 68.5% and 55.4%, respectively. Moreover, under this growth condition the amount of Cr(VI) removed by the biomass of *Aspergillus* sp. N2, *Penicillium* sp. N3 and *Penicillium* sp. N5 was 145 mg/g, 603 mg/g and 46 mg/g, respectively.

To explore possible usefulness of *Aspergillus* sp. N2, *Penicillium* sp. N3, and *Penicillium* sp. N5 for eliminating Cr(VI) from the contaminated soil containing approximately 250 mg Cr(VI)/g soil, we performed two types of bioremediation assays. In the first type of bioremediation assay (slurry-phase assay), Cr(VI) was removed from the contaminated soil directly by each strain (Fig. 6). In this assay the mycelial biomass, obtained from a 3-day culture in PG medium that contained no added Cr(VI), was transferred to the fresh PG liquid medium, followed by the addition of non-sterilized contaminated soil and incubated further at 30°C. Cr(VI) concentration in the control culture medium (Blank) that did not contain any added fungi gradually increased up to 32 mg/L at 72 h post-incubation. However, Cr(VI) concentration in the growth medium inoculated with any one of the three strains slowly increased until 24 h and then remained stationary. Thus, after 72 h of incubation with the *Aspergillus* sp. N2, *Penicillium* sp. N3, and *Penicillium* sp. N5 biomass, the Cr(VI) concentration of the soil samples decreased by about 50%, 60% and 50%, respectively.

In the second type of bioremediation assay (water-phase bioremediation assay), Cr(VI) from the contaminated soil was removed indirectly by *Aspergillus* sp. N2, *Penicillium* sp. N3 and *Penicillium* sp. N5 (Fig. 7). For this purpose the Cr(VI) was first leached out from the contaminated soil

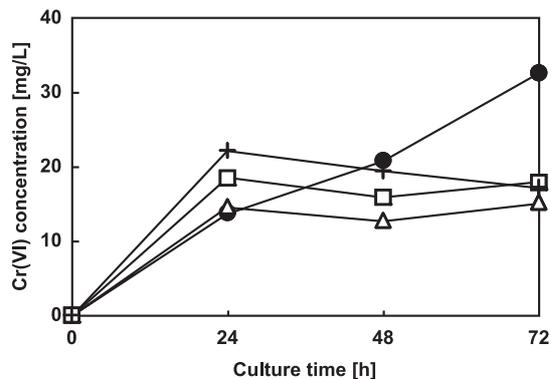


Fig. 6. Time course of changes in Cr(VI) levels in samples from contaminated soil using slurry phase bioremediation.

Symbols represent: —●—, blank; —△—, *Aspergillus* sp. N2; —□—, *Penicillium* sp. N3; and —+—, *Penicillium* sp. N5.

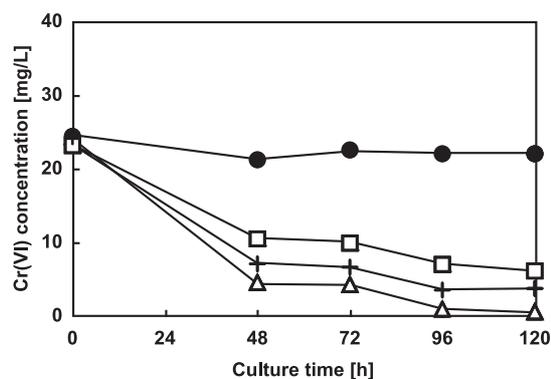


Fig. 7. Time course of changes in Cr(VI) levels in samples from contaminated soil using water phase bioremediation.

Symbols represent: —●—, blank; —△—, *Aspergillus* sp. N2; —□—, *Penicillium* sp. N3; and —+—, *Penicillium* sp. N5.

by shaking with distilled water at pH 8.0 for 24 h. We have observed that the pH of the water used for leaching determined the rate of chromate extraction from the contaminated water, the optimal pH being 8.0 (data not shown). At this pH, 24 h shaking with water extracted out all chromium present in the contaminated soil. The mycelial biomass, obtained from a 3-day culture of one of the fungi in PG medium that contained no added Cr(VI), was then mixed with the chromium-leached water, and fresh PG liquid medium (9 : 1), and the biomass mixture was incubated further at 30°C. The pH of this biomass mixture was approximately 7.5, and the Cr(VI) concentration was approximately 25 mg/L. Fig. 7 shows the time dependent decrease in the Cr(VI) concentration by *Aspergillus* sp. N2, *Penicillium* sp. N3 and *Penicillium* N5. As shown, each strain was able to decrease the Cr(VI) concentration significantly until 48 h (81% by *Aspergillus* sp. N2, 55% by *Penicillium* sp. N3 and 71% by *Penicillium* sp. N5). Beyond 48 h incubation, the Cr(VI) concentration removing ability of each strain was slow. After 120 h incubation, *Aspergillus* sp. N2 decreased the Cr(VI) concentration in the mixture by almost 100%, whereas *Penicillium* sp. N3 and *Penicillium* sp. N5 decreased the Cr(VI) concentration by 74% and 85%, respectively. In the absence of any added fungal strain (control blank), the Cr(VI) concentration in the mixture did not decrease. Then, total Cr concentration did not change at each condition (data not shown).

4. Discussion

Metal-polluted environments pose serious health and ecological risks. Metal containing industrial effluents constitute a major source of metallic pollution. Thus, researchers are continuously searching for naturally occurring microbes having better metallic pollutant transformation capabilities over a wider range of microbial growth conditions. Because of the ever-increasing concerns about the chromate toxicity in chromium deposits we have focused our attention in iso-

lating chromate removing fungi and their use in the detoxification of chromate contaminated soil. The present study describes the isolation of chromate-resistant *Aspergillus* sp. (strain N2) and *Penicillium* sp. (strain N3 and strain N5) strains, which can readily decrease the Cr(VI) concentration in the growth media as well as from contaminated soil samples. These three strains showed high degree of tolerance to chromate (1000 mg/L), which is greater than the previously reported chromate tolerance (25–200 mg/L) of the Cr(VI)-resistant *Aspergillus* sp. Ed8 and *Penicillium* sp. H13 strains¹. Furthermore, the three fungi reported in this study have the ability to grow and also have high activity at low pH (3.0–6.0). In contrast, most reported microorganisms have the high ability to remove chromate at neutral pH^{1,2,5,8,12–15}.

Our other study suggested two mechanisms by which chromate could be removed¹⁰. First, chromate could be reduced to a less toxic lower oxidation state by an enzymatic reaction. Results described here showed Cr(VI) removal ability at acidic pH was lower than at neutral pH. Wang *et al.*³² reported that reduction of chromate to a lower oxidation state by *Enterobacter* strain occurred between pH 6.5–8.5 and the reduction was strongly inhibited at pH 5 and pH 9, while at pH 9.0 *Achromobacter* sp. completely reduced Cr(VI)¹⁶. However, since Cr(VI) reduction is enzyme-mediated, pH changes affects the enzyme ionization rate, changes the protein's conformation and consequently affects the enzyme activity. A coexisting ion could also affect the enzyme activity. At neutral pH condition, the presence of Cu(II) affected the Cr(VI) removal abilities of these strains. Cu(II) enhanced the Cr(VI) removing ability of *Penicillium* sp. N3 at strong acid condition, causing a drop in the Cr(VI) concentration in the culture medium from its initial concentration of 60 mg/L to almost undetectable level after 144 h growth. This result is similar to that observed in *Bacillus* sp. RE expressing a Cr(VI) reducing enzyme, where the enzyme activity was enhanced by Cu(II) and Ni(II) and inhibited by Hg(II)⁹. Cr(VI) removal of *Aspergillus* sp. N2

and *Penicillium* sp. N3 was caused by enzymatic reaction significantly¹⁰. Moreover, the addition of Cu(II) caused the growth of *Aspergillus* sp. N2 was inhibited drastically. The DMW of *Penicillium* sp. N3 and *Penicillium* sp. N5 was also influenced by the presence of Cu(II). Thus, the existence of metallic ion was important to grow fungi. Further studies are necessary to extend our understanding of the effects of the coexisting ions on the Cr(VI) reducing activity of the strains reported in this study.

Biosorption is the second mechanism by which chromate concentration is reduced. The fungal cell wall can be regarded as a mosaic of different functional groups, which could form coordination complexes with metals. Zafar et al.³³ showed biosorption ability of *Aspergillus* strains isolated from wastewater treated soil. At 6 mM initial concentration of Cr (pH 4.5), Cr adsorption value of *Aspergillus* sp. 2 (1.56 mg/g) exceeded the Cr adsorption value (1.20 mg/g) of the less tolerant counterpart *Aspergillus* sp. 1. Some *Penicillium* strains were also reported to adsorb chromium³³. For example, *Penicillium purpurogenum* have the capacity of adsorbing 36.5 mg of Cr(VI) ions per g of fungal biomass²⁷. The chromium sorption by *Aspergillus* sp. N2 and *Penicillium* sp. N3 was confirmed¹⁰. Under acidic condition biosorption of chromium by these strains was higher than at neutral pH. The pH condition has a large influence on chromium sorption of fungi. However, the influence of sorption on mycelia in the chromium removal was little.

Aspergillus niger also has the ability to reduce and adsorb Cr(VI)²⁸. When the initial concentration of Cr(VI) was 50 mg/L, *Aspergillus niger* mycelium removed 8.9 mg of chromium/g dry weight of mycelium in 7 days. In the present study, Cr(VI) removed by the biomass of *Aspergillus* sp. N2, *Penicillium* sp. N3 and *Penicillium* sp. N5 were 111 mg/g, 143 mg/g, and 106 mg/g, respectively. Thus, the chromium removal abilities of these strains are equal or better than the other reported strains.

Aspergillus sp. N2, *Penicillium* sp. N3 and *Penicillium* sp. N5 were able to decrease the initial Cr(VI) concentrations of the contaminated soils. While Cr(III) compounds are very stable in soils, Cr(VI) is very unstable and is easily mobilized in both acidic and alkaline soils³⁴. Since Cr(VI) could be easily extracted from the soil into the aqueous solution, we employed two types of bioremediation assays (slurry phase bioremediation and water phase bioremediation) to determine the effectiveness of the isolated strains in removing Cr(VI) from the contaminated soils. In addition, we adopted seed culture in these assays because inoculated strains must grow preferentially. Our results clearly showed that the fungal strains *Aspergillus* sp. N2, *Penicillium* sp. N3 and *Penicillium* sp. N5 succeeded in reducing the Cr(VI) concentration in non-sterilized soils. These results suggest the potential applicability of *Aspergillus* sp. N2, *Penicillium* sp. N3 and *Penicillium* sp. N5 for the remediation of Cr(VI) from the contaminated soil in the fields.

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References

- 1) Acevedo-Aguilar, J.F., E.E. Espino-Saldana, L.I. Leon-Rodriguez, E.M. Rivera-Cano, M. Avila-Rodriguez, K. Wrobel, K. Wrobel, P. Lappe, M. Ulloa, and F.J. Gutierrez-Corona. 2006. Hexavalent chromium removal in vitro and from industrial wastes, using chromate-resistant strains of filamentous fungi indigenous to contaminated wastes. *Can. J. Microbiol.* 52: 809–815.
- 2) Baldi, F., A.M. Vaughan, and G.J. Olson. 1990. Chromium (VI)—resistant yeast isolated from a sewage treatment plant receiving tannery waste. *Appl. Environ. Microbiol.* 56: 913–918.
- 3) Bartlett, R., and B.R. James. 1998. Mobility and bioavailability of chromium in soils. *Adv. Environ. Sci. Technol.* 20: 267–304.
- 4) Biedermann, K.A., and J.R. Landolph. 1990. Role of valence state and solubility of chromium compounds on induction of cytotoxicity, mutagenesis, and anchorage independence in diploid human fibroblasts. *Cancer Res.* 50: 7835–7842.
- 5) Bingol, A., H. Uzun, Y.K. Bayhan, A. Karagunduz, A. Cakici, and B. Keskinler. 2004. Removal of chromate anions from aqueous steam by a cationic surfactant-modified yeast. *Biore-sour. Technol.* 94: 245–249.
- 6) Cervantes, C., J. Campos-Garcia, S. Devars, S.F. Gutierrez-Corona, H. Loza-Tavera, J.C. Torres-Guzman, and R. Moreno-Sanchez. 2001. Interactions of chromium with microorganisms and plants. *FEMS Microbiol. Rev.* 25: 335–347.
- 7) Cervantes, C., and S. Silver. 1992. Plasmid chromate resistance and chromate reduction. *Plasmid.* 27: 65–71.
- 8) Dias, M.A., I.C. Lacerda, P.F. Pimentel, H.F. de Castro, and C.A. Rosa. 2002. Removal of heavy metals by an *Aspergillus terreus* strain immobilized in a polyurethane matrix. *Lett. Appl. Microbiol.* 34: 46–50.
- 9) Elngovan, R., S. Abhipsa, B. Rohit, P. Ligy, and K. Chandraraj. 2006. Reduction of Cr(VI) by a *Bacillus* sp. *Biotech. Lett.* 28: 247–252.
- 10) Fukuda, T., Y. Ishino, A. Ogawa, K. Tsutsumi, and H. Morita. 2008. Cr(VI) reduction from contaminated soils by *Aspergillus* sp. N2 and *Penicillium* sp. N3 isolated from chromium deposits. *J. Gen. Appl. Microbiol.* in press.
- 11) Fruchter, J. 2002. In situ treatment of chromium contaminated groundwater. *Environ. Sci. Technol.* 36: 464–472.
- 12) Gadd, G.M., and C. White. 1993. Microbial treatment of metal pollution: a working biotechnology. *Trends Biotechnol.* 11: 353–392.
- 13) Kamaludeen, S.P., M. Megharaj, A.L. Juhász, N. Sethunathan, and R. Naidu. 2003. Chromium microorganism interactions in soil: remediation implications. *Rev. Environ. Contam. Toxicol.* 178: 93–164.
- 14) Kaszycki, P., D. Fedorovych, H. Ksheminska, L. Babyak, D. Wojcik, and H. Koloczek. 2004. Chromium accumulation by living yeast at various environmental conditions. *Microbiol. Res.* 159: 11–17.
- 15) Kirk, M.P., F.P. Cannon, C.J. David, and A.J. Stalpers. 2001. *Dictionary of the fungi.* CABI Publishing, UK.
- 16) Lowe, K.L., W. Straube, B. Little, and J. Jones-Meehan. 2003. Aerobic and anaerobic reduction of Cr(VI) by *Shewanella oneidensis*. *Acta. Biotechnol.* 23: 161–178.
- 17) Ma, Z., W. Zhu, H. Long, L. Chai, and Q. Wang. 2007. Chromate reduction by resting cells of *Achromobacter* sp. Ch-1 under aerobic conditions. *Process. Biochem.* 42: 1028–1032.
- 18) Mergesin, R., and F. Schinner. 1996. Bacterial heavy metal tolerance extreme tolerance to nickel in *Arthrobacter* sp.. *J.*

- Basic. Microbiol. 36: 269–282.
- 19) Michel, C., M. Brugna, C. Aubert, A. Bernadac, and M. Bruschi. 2001. Enzymatic reduction of chromate, comparative studies using sulphate reducing bacteria, Key role of polyheme cytochrome c and hydrogenases. Appl. Microbiol. Biotechnol. 55: 95–100 .
 - 20) Muter, O., A. Patmalnieks, and A. Rapoport. 2001. Interrelations of the yeast *Candida utilis* and Cr(VI): metal reduction and its distribution in the cell and medium. Process Biochem. 36: 963–970.
 - 21) Ortel, J.W., E.D. Staren, L.P. Faber, W.H. Warren, and D.P. Braun. 2002. Modulation of tumor infiltrating lymphocyte cytolytic activity against human non small cell lung cancer. Lung Cancer. 36: 17–25.
 - 22) Park, C.H., M. Keyhan, B. Wielinga, S. Fendorf, and A. Matin. 2000. Purification to homogeneity and characterization of a novel *Pseudomonas putida* chromate reductase. Appl. Environ. Microbiol. 66: 1788–1795.
 - 23) Pepi, M., and F. Baldi. 1992. Modulation of chromium (VI) toxicity by organic and inorganic sulfur species in yeasts from industrial wastes. Biometals. 5: 179–185.
 - 24) Pillichshammer, M., T. Pumpel, R. Poder, K. Eller, J. Klima, F. Schinner. 1995. Biosorption of chromium to fungi. Biometals. 8: 117–121.
 - 25) Ramirez-Ramirez, R., C. Calvo-Mendez, M. Avila-Rodriguez, P. Lappe, M. Ulloa, R. Vazquez-Juarez, and J.F. Gutierrez-Corona. 2004. Cr(VI) reduction in a chromate-resistant strain of *Candida maltose* isolated from the leather industry. Antonie. Van. Leeuwenhoek. 85: 63–68.
 - 26) Reeves, M.W., L. Pine, J.B. Neilands, and A. Balows. 1983. Absence of siderophore activity in *Legionella* species grown in iron-deficient media. J. Bacteriol. 154: 324–329.
 - 27) Say, R., N. Yilmaz, and A. Denizli. 2004. Removal of chromium (VI) ions from synthetic solutions by the fungus *Penicillium purpurogenum*. Eng. Life Sci., 4: 276–280.
 - 28) Srivastava, S., and S.I. Thakur. 2006. Biosorption potency of *Aspergillus niger* for removal of chromium (VI). Current Microbiol. 53: 232–237.
 - 29) Shrivastava, S., and S.I. Thakur. 2003. Bioabsorption potentiality of *Acinetobacter* sp. Strain IST103 of a bacterial consortium for removal of chromium from tannery effluent. J. Sci. Ind. Res. 62: 616–622.
 - 30) Taylor, M.M., E.J. Diefendorrf, and G.C. Na. 1990. Enzymatic treatment of chrome shavings. J. Am. Leather Chem. Assoc. 85: 264–275.
 - 31) Vala, A.K., N. Anand, P.N. Bhatt, and H.V. Joshi. 2004. Tolerance and accumulation of hexavalent chromium by two seaweed associated *Aspergilli*. Mar. Pollut. Bull. 48: 983–985.
 - 32) Wang, P., T. Mori, K. Toda, and H. Ohtake. 1990. Membrane-associated chromate reductase activity from *Enterobacter cloacae*. J. Bacteriol. 172: 1670–1672.
 - 33) Zafar S., F. Aqil, and I. Ahmadf. 2007. Metal tolerance and biosorption potential of filamentous fungi isolated from metal contaminated agricultural soil. Biores. Technol. 98: 2557–2561.
 - 34) Zayed, A., and N. Terry. 2003. Chromium in the environment: factors affecting biological remediation. Plant and Soil. 249: 139–156.