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Chromium Reduction and Removal Using Fungi Isolated from Tannery-Effluent Polluted Soil

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REMOVAL of excess heavy metal ions from wastewater is essential for reducing their extreme toxicity. One metal of major concern is chromium. Cr(VI) and Cr(III) are the principal forms of chromium found in natural waters. Six fungal cultures, isolated from tannery effluent-polluted soils were able to grow in medium containing 10–200 mg/l Cr(VI) as $K_2Cr_2O_7$. The growth response among the six fungi at different concentrations of Cr(VI) varied greatly. Fungal biomass decreased with increased Cr(VI) concentrations. All fungal cultures were able to reduce Cr(VI) to Cr (III) at 10, 25 and 50 mg Cr(VI)/l. One of the isolates was capable of tolerating up to 200 mg Cr (VI)/l and was able to reduce Cr(VI) up to 64.6%. This isolate was identified as an *Aspergillus tamarii* by 18S rRNA gene sequence homology. The ability of this fungus to remove chromium from the environment was established and demonstrated its potential use for the *in situ* detoxification of Cr(VI)-contaminated waste streams.

Keywords: Tannert-effluent, Hexavalent chromium, Fungi, Chemical modification, Biosorption.

The detoxification of the heavy metal contaminants in wastewater and industrial effluents is of great significance owing to the growing problems of limited potable water supply. Hexavalent chromium [Cr(VI)] compounds are being used in a wide variety of commercial processes and unregulated disposal of the chromium containing effluent in both developing and developed countries has led to the contamination of soil, sediment, surface and ground waters (Szulczewski *et al.*, 1997). Cr(VI), one of the acute carcinogens is released in electroplating, textile dyeing and tanning effluent, and may accumulate to concentrations higher than the internationally recommended value of 0.05 mg/l (Nisha and Pandey, 1984).

Chromate reduction to trivalent chromium [Cr(III)] is a potential detoxification process that could be achieved via chemical or biological methods. However,

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chemical reduction requires energy input and large quantities of chemicals (Srivastava *et al.*, 1986). Therefore, biological reduction of chromate could provide a more economical alternative. Many microorganisms have been reported to reduce the highly soluble and toxic hexavalent chromium to the less soluble and less toxic trivalent ion (Cross *et al.*, 1997 ; Ganguli & Tripathi, 2001 and Pattanapipitpaisl *et al.*, 2001). However, the potential for biological treatment of Cr(VI)-contaminated waste is limited because some microorganisms lose viability in the presence of high concentrations of chromate (Vala *et al.*, 2004). Isolating chromate-reducing fungi from tannery-effluent polluted environments could, therefore, be useful.

In concept of biosorption, several chemical processes may be involved, such as adsorption, ion exchange, and covalent bonding with the biosorptive sites of the microorganisms including carboxyl, hydroxyl, sulphydryl, amino and phosphate groups (Kapoor *et al.*, 1999; Say *et al.*, 2001 and Bai & Abraham, 2002). Fungal cell walls and their components have a major role in biosorption (Hafez *et al.*, 1997 and Kapoor & Viraraghavan, 1997). The purpose of this study was screen fungal isolates from tannery-effluent polluted soils, to assess their chromate reduction capacities at a selected concentration of Cr(VI). In addition, the objective was to obtain information about the enhancement of chromium biosorption using a chemically modified biomass of *A. tamrrii.*

Material and Methods

Fungal isolation, identification and maintenance

Fungal cultures were isolated from tannery effluent polluted soils collected nearby the leather complex industry at Max, Alexandria, Egypt. Fungi were detected and isolated using the dilution plate method: dilutions of 10^{-1} or 10^{-3} were prepared and 1 ml was plated on Dextrose peptone agar (DPA) (Martin, 1950). Five plates for each dilution were used and were incubated at 30 °C. The resulting colonies were counted usually after 5 to 7 days. The average number of colonies was multiplied by the dilution factor to obtain the number in the original soil sample based on oven dry weight of the soil. The developing fungal colonies were identified up to the species level using morphological characters some that were determined by microscopy (Gilman, 1957 and Moubasher, 1993). Further identification of the highest chromate tolerant fungal isolate was by 18S rRNA gene sequencing. Genomic DNA was isolated according to the method of Dellaporta (1994) and the a 581-pb fragment of the 18S rRNA gene was amplified by PCR using fungus-specific primers TR1 5-GTTTCTAGGACCGCCGTA-3 at position 834 and TR2 5-CTCAAACTTCCATCGACTTG-3 at position 1415 (Bock *et al.*, 1994). The presence of a single PCR product were verified by electrophoresis in 2 % w/v agarose gels visualizing ethidium bromide-stained DNA with a gel documentation system. The PCR products were purified with a QIA quick spin PCR purification kit (Qiagen, Hilden, Germany) and were then used for sequence analysis with a Perkin Elmer 373 DNA Sequencer, in combination with ABI PRISMe dye deoxy terminator cycle sequencing kit (Applied Biosystems, Foster Vity CA). The sequences obtained were compared

with 18S rRNA gene sequences in GenBank and ribosomal RNA databases. The fungal isolates were maintained on DPA at 30 °C and were stored as micro-conidial suspensions in 50 % glycerol at -80 °C.

Fungal growth and Cr (VI) tolerance screen

The chromate tolerance of six fungal isolates (A- *Aspergillus niger*, B-*Aspergillus fumigatus*, C- *Aspergillus flavus*, D- *Aspergillus tamarii*, E-*Penicillium chrysogenum* and F- *Rhizopus nigricans*) were determined by measuring the mycelial biomass. The fungal cultures were grown in 250 ml Erlenmeyer flasks containing 100 ml of Sabouraud liquid medium (SPD) (Errasquin and Vazquez, 2003), which consisted of 1% peptone and 2% dextrose, pH 5.8. Peptone, rather than other nitrogen-containing organic substrates, was used because of its comparatively low metal binding capacity (Garcia-Toledo *et al.*, 1985). The SPD medium was supplemented with 10, 25, 50, 100, 150, 200 and 250 mg/l of Cr(VI) as $K_2Cr_2O_7$ (Aldrich, WI). The media were inoculated with fungal spores removed from the margins of 7 days old colonies and then were incubated at 28 °C on an orbital shaker at 150 rpm for 6 days. The mycelial masses were harvested by filtration, washed thoroughly with de-ionized distilled water and were dried overnight at 65 °C to determine the dry weight. The culture broths were retained in order to measure the Cr(VI) concentration in the filtrates. Three replications of all assays were performed.

Analysis of Cr(VI) ions

Chromate-reducing activity was estimated as the decrease in chromate concentration in the supernatant. Chromium (VI) content in samples were determined by measuring optical density of the purple complex of Cr(VI) with 1,5-diphenylcarbohyrazide at 540 nm by UV spectrophotometry (Pattanapipitpaisal *et al.*, 2001).

Biomass production

The highest chromate tolerant fungus, *Aspergillus tamarii* was cultivated in liquid medium using the shake flask method. Spores and mycelium from the DPA spread plate cultures were transferred to 500 ml Erlenmeyer flasks containing 200 ml growth medium. This growth medium had the following composition (g/l): dextrose, 20; peptone, 10; NaCl, 0.2; $CaCl_2 . 2H_2O$, 0.1; KCl, 0.1; K_2HPO_4, 0.5; $NaHCO_3$, 0.05; $MgSO_4$, 0.25; $Fe(SO_4)_2.7H_2O$, 0.005. The pH of the growth medium was adjusted to 5.0 using 1 N HCl before autoclaving. Once inoculated, flasks were shaken on a rotary shaker at 150 rpm for five days at 30 °C. The dry weight of the biomass was determined after washing the harvested mycelia twice with de-ionized water and drying it to a constant weight at 105 °C. Subsequently the dry mycelia were treated with two different chemical combinations prior to the biosorption experiments. The first was an acid/alkali extraction using 0.1 N solutions of mineral acids (HCl and H_2SO_4) and alkali (NaOH) selected (Bai and Abraham, 2002). Five grams of the biomass powder was mixed with 500 ml of each of the acid/alkali solution and was agitated at 120 rpm for 24 hr. The second was a wash in alcohol and acetone, Biomass powder (I g) was treated with 100 ml of 50% (v/v) each of ethyl alcohol, methyl alcohol and acetone, the suspension was agitated for 24 hr and then was

filtered, washed and dried. After treatment, the powdered biomass was filtered, washed with generous amounts of de-ionized distilled water till the pH of the wash solution was in the near-neutral range (7.0 – 7.2) and then dried over night in an oven at 60 °C.

Biosorption studies

Cr(VI) and Cr(III) solutions (100 mg/l) were treated with known quantities of biosorbent and the adsorption capacity (mg Cr/g biomass) was determined. A biomass dose of 0.2% (w/v), a Cr solution of pH 3.4, and an ambient temperature (30°C), were determined to be optimal in our preliminary studies, and were subsequently used in all biosorption experiments. After acid digestion (HCl/HNO_3, 4:1, v/v) (McGrath and Cunliffe, 1985), chromium concentrations absorbed with fungal biomass was determined using an atomic absorption spectrophotometer. For the experiment, the unmodified/ native biomass powder was used as controls.

Results and Discussion

Fungal isolation and identification

Heavy metal contamination of soils originating from industrial activities is one of the major environmental problems in many parts of the world (Gremion *et al.*, 2004). Heavy metals affect all groups of organisms and ecosystem processes, including microbial activities (Giller *et al.*, 1998). The soil from the tannery waste disposal site used for isolation of fungi contained 44.9 mg Cu kg^{-1}, 124 mg Pb kg^{-1}, 208 mg Zn kg^{-1} and 16 865 mg Cr kg^{-1} dry soil and a total culturable fungal count of 3.4×10^2 c.f.u. g^{-1} (Table 1). This relatively low fungal count can be attributed to the presence of heavy metals in such high concentrations. Six fungal isolates A, B, C, D, E and F were selected based on colony morphology and were identified as *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus sp.*, *Penicillium chrysogenum* and *Rhizopus nigricans*, respectively, based on morphological characters.

TABLE 1. Total culturable fungi and physicochemical characteristics of tannery-effluent polluted soils.

Soil properties	Tannery effluent polluted soil
Soil texture	S.L*
CEC** (meq $100g^{-1}$)	34.8
pH (H_2O)	6.8
Organic matter (%)	2.6
Total Cr (mg kg^{-1})	16865
Mobile*** Cr (mg kg^{-1})	75.4
Total Cu (mg kg^{-1})	44.9
Mobile Cu (mg kg^{-1})	1.1
Total Pb (mg kg^{-1})	124.5
Mobile Pb (mg kg^{-1})	22.2
Total Zn (mg kg^{-1})	208
Mobile Zn (mg kg^{-1})	6.9
CFU of culturable fungi/g soil	3.4×10^{-2}

S= Sandy L= Loam; CEC= Cation Exchange Capacity; Mobile= Water extractable metal

Effect of Cr(VI) on fungal growth

Metals influence microorganisms by adversely affecting their growth, morphology and biochemical activities, resulting in a decrease in their biomass and numbers (Giller *et al.*, 1998). However, some microorganisms have evolved mechanisms of resistance that has led to their selection (Losi and Frankenberger, 1994). Figure 1 presents the results from batch experiments conducted to study the effect of Cr(VI) on the viability of *A. niger*, *A. fumigatus*, *A. flavus*, *Aspergillus sp.*, *P. chrysogenum* and *R. nigricans* cells exposed to varying concentrations of Cr(VI) while they were growing. All six fungal isolates were able to grow on SPD-broth medium supplemented with 100 mg Cr(VI)l^{-1}, but not with 250 mg Cr(VI)l^{-1} medium indicating the same degree of Cr(VI) resistance. The growth response of the six fungi with different concentrations of Cr(VI) varied greatly. In general, fungal growth rate (dry weight) decreased with the increase of Cr(VI) concentrations. Only the growth of fungal isolate D (*Aspergillus sp.*) was not affected by Cr(VI) up to a concentration of 200 mg l^{-1} compared with the other five fungal isolates. However, at 200 mg Cr(VI)l^{-1} the growth dramatically decreased by an 80% reduction, while no growth was detected at 250 mg l^{-1} (Fig. 1). Toxicity that caused reduction in biomass concentration was found to increase with increasing Cr(VI) concentration; implying the occurrence of denaturation of biopolymers capable of complexing Cr(VI), resulting in inhibition, decreased viability, and even cell death and lysis (Chang *et al.*, 1995). Dursun *et al.* (2003) reported that *Aspergillus niger* growth was very sensitive to all concentrations of Cr (VI). The increase of Cr(VI) concentration from 25 to 50 mg dm^{-3} lead to a drastic decrease of specific growth rate from 0.075 to 0.039 h^{-1}.

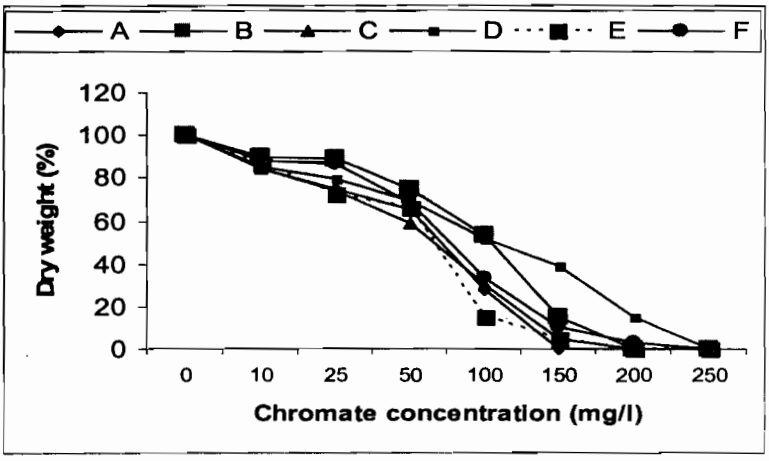

Fig.1. Effect of Cr(VI) concentrations on : A- *Aspergillus niger*, B- *Aspergillus fumigatus*, C- *Aspergillus flavus*, D- *Aspergillus tamarii*, E- *Penicillium chrysogenum* and F- *Rhizopus nigricans* growth .

Chromate reduction

All six fungal isolates were used in further experiments. The effect of Cr(VI) concentration on chromate reduction was investigated under aerobic conditions over the range 10 – 250 mg l^{-1} of Cr(VI) as $K_2Cr_2O_7$. Chromate reduction was reduced even at the highest Cr(VI) concentration. The six fungal isolates were capable to completely reduce all the chromate reduction from the media amended with 10, 25 and 50 mg Cr(VI)l^{-1} (Fig. 2). The highest rate of chromate removal was observed with *Aspergillus sp.* (99.4%, 98% and 64.6%), at 100, 150 and 200 mg Cr(VI)l^{-1}, respectively. The biotransformation of orange colour to blue colour as shown in Fig. 3 indicated the ability of *Aspergillus sp.* to reduce hexavalent chromium Cr(VI) to trivalent chromium Cr(III). The concentration of the Cr(VI) remaining was measured by phenylcarbazide testing.

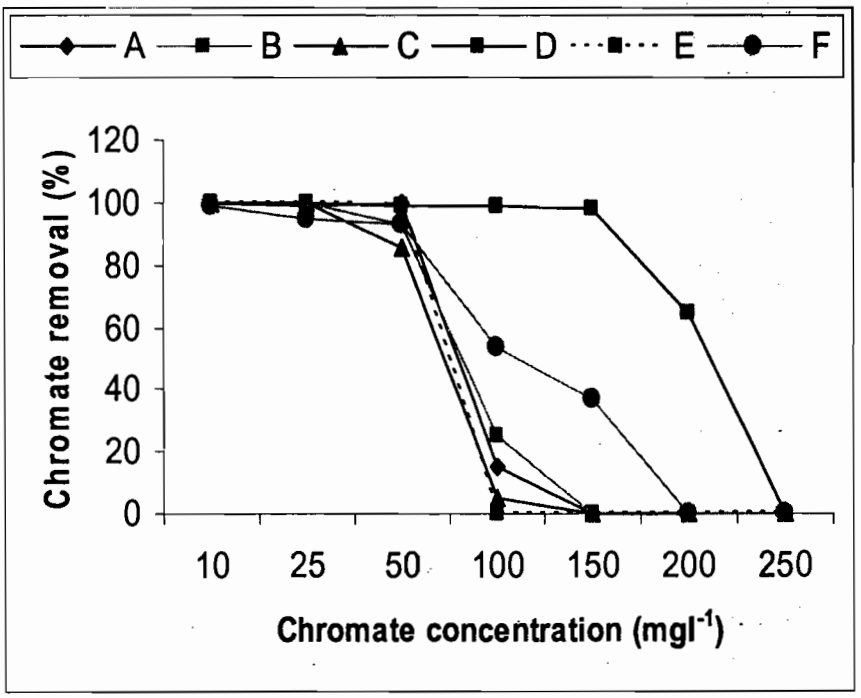

Fig. 2. Percent of chromate removal by: A- *Aspergillus niger*, B- *Aspergillus fumigatus*, C- *Aspergillus flavus*, D- *Aspergillus tamarii*, E-*Penicillium chrysogenum* and F- *Rhizopus nigricans* grown on different Cr(VI) concentrations.

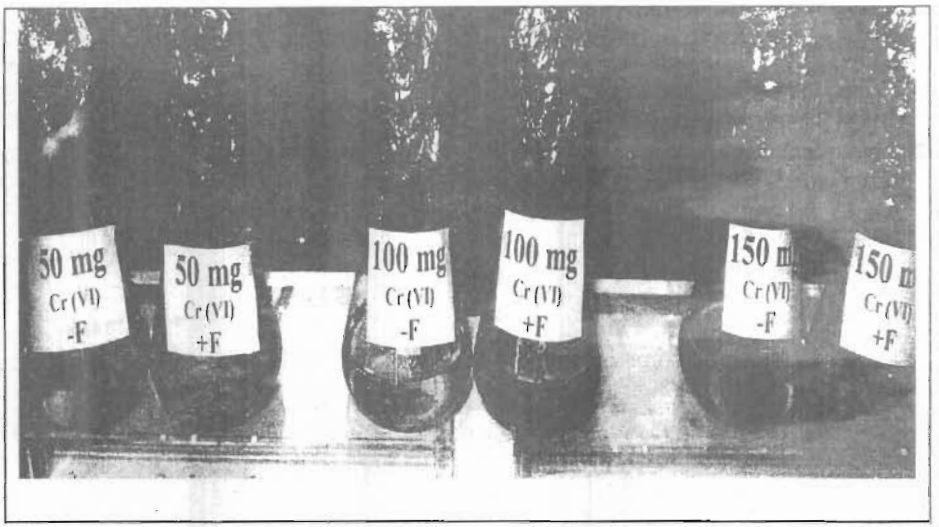

Fig. 3. Fungal growth on different chromate concentrations, blue colour in flask inoculated with *A. tamarii* indicate the biotransformation of Cr(VI) to Cr(III).

Chromium exists in several oxidation states, but the most stable are the trivalent Cr (III) and hexavalent Cr(VI) forms that have different chemical characteristics and biological effects (Cervantes *et al.*, 2001). The ability of chromate resistant bacteria to reduce Cr(VI) is widely recognized and is not an exclusive characteristic of specific bacterial groups or populations (Srinath *et al.*, 2002; Paul & Pal, 2004; Abou-Shanab *et al.*, 2005 and Camargo *et al.*, 2005). Francisco *et al.* (2002) concluded that Cr(VI) resistance and reduction are both shared abilities, probably reflecting horizontal genetic transfer of the determinants resulting from selective pressure in environments contaminated with Cr(VI). This ability enables microbial populations to adapt and evolve rapidly in response to stress resulting from changes in the environments.

18S rRNA sequence analysis

The fungal isolate *Aspergillus sp.* (D), with the highest capability of reducing chromate, was selected for 18S rRNA gene sequence analysis. Genomic DNA of this fungus was isolated and the fungus-specific primers TR1 and TR2 were used to amplify a 581-bp fragment within the small ribosomal subunit (Fig. 4). From the 18S rRNA sequences it was concluded that fungal isolate –D- was closely related to *Aspergillus tamarii* based on 91% sequence similarity. The nucleotide sequence coding for 18S rRNA gene of *A. tamarii* strain D has been submitted to the GenBank database under accession number DQ234302.

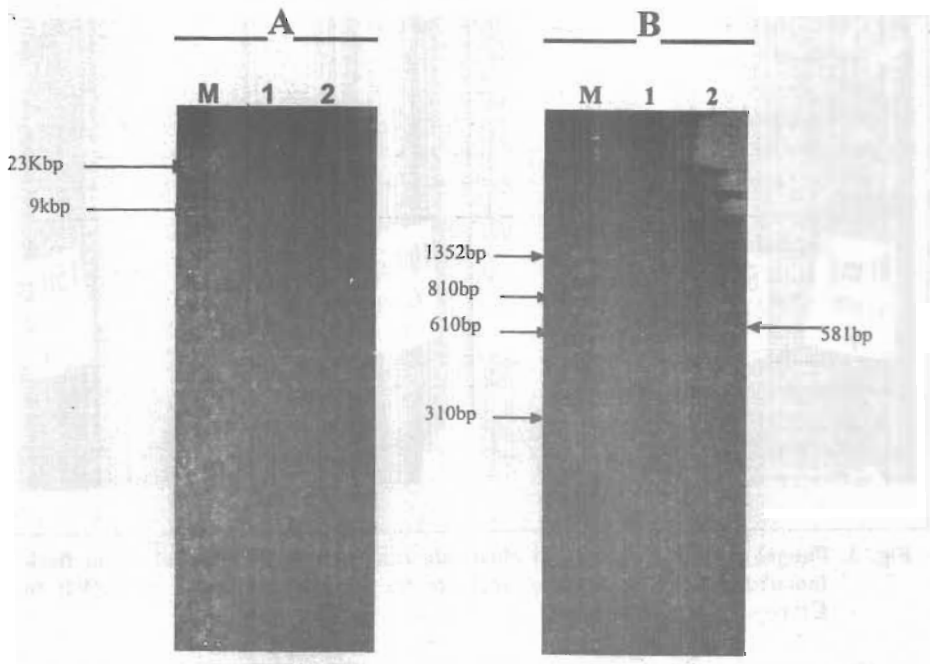

Fig. 4. A- Agrose gel 2% showed genomic DNA from isolated fungus, Lane 1: Lambda DNA *HindIII* marker, Lane 2 and 3: The genomic DNA and B-Electrophoresis of the products amplified from fungal isolate, Lane M: MVL DNA marker, Lane 1 and 2: The 581bp PCR product.

Chromium biosorption by chemically modified biomass of A. tamarii

The potential of living and dead biomass of microbial/plant origin to adsorb heavy metal ions from solutions has been reported (Bailey *et al.*, 1999 and Volesky & Holan, 1995). This technology is a passive method of metal removal by dead biomass. The non-living biomass of fungi (Pumpel and Schinner, 1993) has been reported for effective and economical removal of a variety of toxic heavy metals from waste water. The biosorptive removal of metal ions from aqueous solution mainly depends on chemical mechanisms involving the interaction of metal ions with specific groups associated with the biosorbent cell wall. In our examination with *A. tamarii* biomass, the pretreatment with ethanol, hydrochloric acid and sodium hydroxide enhanced Cr(III) adsorption by 9, 7, 5 and 3-fold, respectively, compared with the untreated control. *A. tamarii* biomass treated with acetone enhanced Cr(VI) adsorption by 1.2 fold over the untreated control (Fig. 5) . The dissimlar results achieved with the different treatments could be attributed to acid hydrolysis yielding relatively pure amino sugar (Nair and Madhavan, 1992), which is more easily protonated at adsorption pH. Thus extraction of finely powdered biomass in acid could expose more binding sites and, therefore, enhance the accessibility of the sorbate ions to the sorption sites (Teles *et al.*, 1997). Treatment of the biomass with 0.1N NaOH caused hydrolysis of protein constituents and also resulted in deacetylation of chitin. The

improved biosorption potential of *Aspergillus uvarum* after alkali treatment for cations have been reported by Luef *et al.* (1991) and Fourest & Roux (1992). Their interpretation was that alkali treatment resulted in the exposure of certain chemical groups enhancing the binding of cationic metal contaminants. However, in our present investigation, Cr(VI) in aqueous solution is in a negatively charged oxo anion complex form and, thus, is bound to the chitin rich surface by a different mechanism probably resulting in the negative trend.

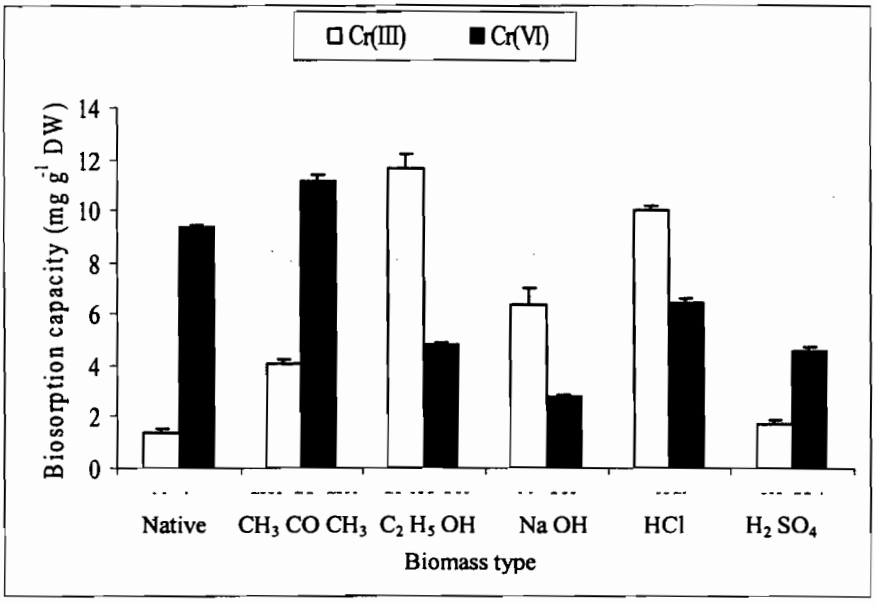

Fig. 5. The metal biosorptive capacity of native and chemically pretreated *A. tamrii* biomass. (Cr concentration 100 mg l^{-1}; pH= 3.4; Biomass dose= 0.2%, w/v). The bar in the top of column is the standard deviation.

Conclusion

Six fungal isolates from tannery-effluent polluted soils were able to grow in medium containing 10-200 mg Cr(VI)l^{-1}. These fungal isolates were identified as *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus tamarii*, *Penicillium chrysogenum* and *Rhizopus nigricans*. The Cr(VI) content of the medium, supplemented with 10 mg/l $K_2Cr_2O_7$, disappeared completely after 6 days growth of *A. niger*, *A. fumigatus*, *A. flavus* and *A. tamarii*. *A. tamarii* was able to reduce chromate by 100%, 99.6%, 99.4%, 99.4%, 98% and 64.6% at 10, 25, 50, 100, 150 and 200 mg Cr(VI)l^{-1}, respectively. Biomass of *A. tamarii* treated with acetone enhanced Cr(VI) adsorption by 1.2-fold greater than native biomass. The present study indicated that *A. tamari* has the potential for chromate reduction and appears to be useful as a living biosorbent for removing Cr(III) and Cr(VI) from wastewaters.

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اختزال الكروم وإزالته باستخدام فطريات معزولة من تربة ملوثة بالمخلفات السائلة لمدابغ الجلود

رضا عبد العزيز أبراهيم أبوشنب[1] و السيد السيد حافظ[2]
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أنه من الضرورى بمكان إزالة العناصر الثقيلة من المياه الملوثة وذلك لكمرحدة سميتها للأحياء المائية وللأنسان. ومن بين هذة العناصر التى لها أولوية الاهتمام عنصر الكروم وذلك لخطورته الشديدة أذ أنه يوجد فى صورتين أكثر شيوعا فى المياه الطبيعية ، وهما الكروم الثلاثى والسداسى. وللتخلص من هذا العنصر فقد تم عزل ستة أنواع من الفطريات من تربة ملوثة بمخلفات مدابغ الجلود واتضح من الدراسة أن هذه الفطريات لها القدرة على تحمل من ١٠ -٢٠٠ ملليجرام كروم سداسى. وأن أستجابة نمو هذه الفطريات لهذه التركيزات تختلف من فطر للأخر. أذ أن وزن الكتلة الحية لهذه الفطريات يقل مع زيادة تركيزالكروم السداسى. وقد وجد أن الفطريات الستة لها القدرة على أختزال الكروم السداسى وتحويله الى كروم ثلاثى عند تركيزات ١٠ ، ٢٥ و٥٠ ملليجرام كرومات فى اللتر. وأتضح أيضا من الدراسة أنه من بين هذه الفطريات فطر له القدرة على تحمل ٢٠٠ ملليجرام / لتر كروم سداسى وأنه قادر على أختزاله وتحوله الى كروم سداسى بنسبة ٦٤,٦ ٪ . وقد تم التعرف على نوع هذا الفطر بقراءة التتابع النيوكلوتيدى لجين الريبسوم ١٨ أس مع عقد مقارنة بين هذه التتابعات والتابعات المنشورة فى البنك الجينى. وقد تم دراسة زيادة قدرة هذا الفطرعلى الأدمصاص الحيوى للكروم باستخدام المعاملات الكيميائية. ويتضح من هذه الدراسة أن قدرة هذا الفطرعلى اختزال الكروم وأدمصاصه يمكن أن تستخدم فى إزالة سميته ومعالجة المياه الملوثة به.