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ENHANCEMENT OF Cr^{6+} REMOVAL BY *ASPERGILLUS NIGER* RH19 USING A BIOFERMENTER

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Abstract

Microbial bioremediation is an emerging technology for environmental cleanup. Microorganisms are advantageous for metal detoxification as they are easy to grow, resulting in a rapid production of biomass, and are part of natural environment. In the present study, Cr^{6+} biosorption potentials of four bacterial isolates (*Bacillus* sp. RH69, *Bacillus* sp. RH70, *Bacillus pumilis* RH84 and *Bacillus fumus* RH109), and two fungal isolates, *Aspergillus niger* strains, RH17 and RH19, were determined. *Bacillus fumus* RH109 showed maximum tolerance against Cr^{6+} of 1000 mg/L and *Aspergillus niger* RH19 exhibited resistance up to 275 mg/L. *Bacillus* sp. RH69, in VB mineral salt media, containing 25 mg/L Cr^{6+}, showed 42.60% removal at 30°C (pH 5.0) within 24 hours, but soon after that desorption took place. On the other hand, *Aspergillus niger* RH19 removed 46.00% Cr^{6+} from the same initial concentration of Cr^{6+} at identical pH, temperature and agitation. Growth conditions for this strain were optimized. In batch biosorption studies, *Aspergillus niger* RH19 removed 74.00% Cr^{6+} at 30°C with pH 8.0 from aqueous solution containing 25 mg/L Cr^{6+} at 100 rpm agitation. A pilot study was then conducted to scale up biosorption of Cr^{6+} using stirred tank biofermenter. Cr^{6+} removal by *Aspergillus niger* RH19 was recorded as 60.00% at pH 6.0, 35°C, 100 rpm agitation and 5% DOT (dissolved oxygen tension). These results are promising in terms of development and design of bioreactors for removal of hazardous heavy metal ions from industrial waste water, according to indigenous industrial requirements.

Introduction

Hexavalent chromium, Cr^{6+}, is a widely recognized mutagen and carcinogen associated with various forms of cancers, particularly respiratory tract and pancreatic cancer (Ganguli & Tripathi, 2002). Conventional processes used for removal of heavy metals from industrial waste waters include chemical precipitation, oxidation reduction, filtration, electrochemical techniques and sophisticated separation processes using membranes. However, these processes are usually expensive, and environmentally invasive. During the recent era of environmental protection, the use of microorganisms for the recovery of metals from waste streams, as well as employment of plants for landfill applications, has generated growing attention (Kotrba & Ruml, 2000). There are a wide variety of microorganisms, encompassing bacteria, fungi, yeast, and algae, that can interact with metals and radionuclides through several mechanisms to transform them (Volesky, 1994; Kapoor & Viraraghavan, 1998). Fungi can also be easily grown in substantial amounts using unsophisticated fermentation techniques and inexpensive growth media (Kapoor *et al.*, 1999). Biosorption can be defined as metabolism-independent adsorption of pollutants on microbial biomass, based on the partition process (Ringot *et al*., 2006). Therefore, biosorption carried out by fungi could serve as an economical means of treating effluents charged with toxic metallic ions.

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Leather tanning is one of the main sectors in Pakistan's leather industry. About 90% of its products are exported in finished from, and in Pakistan there are some 600 tanneries in the formal sector, and an equally large number of tanneries in the informal sector. Leather tanneries in Pakistan produce all three categories of waste: waste water, solid waste and air emissions. However, waste water is by far the most important environmental challenge being faced by Pakistan's tanneries (Iqbal *et al*., 1998; Khwaja, 2000). Trivalent chromium salts (Cr^{3+}), are efficiently used as tanning agents in leather industry. A great deal of chrome is released in retaining, dyeing and fat liquoring floats. Spent chrome tanned liquor is greenish in colour, and lightly acidic in nature because of the uses of different acidic chemicals. The industrial effluents containing chromium compounds in hexavalent form are released directly or indirectly into natural water sources, mostly without proper effluent treatment (Shakoori *et al*., 2000). There are a number of reports on increased levels of Cr^{6+} in water bodies and soil near tanneries in Pakistan, therefore, there is an urgent need to develop remedial measures.

In the present study, different *Bacillus* sp., and *Aspergillus niger* isolates chosen after determination of maximum resistivity level against hexavalent chromium, were further accessed for their Cr^{6+} biosorptive capability for metal removal from aqueous solution. The most promising strain, with regard to metal removal (*Aspergillus niger* RH19), was then used to scale up the process in a biofermentor, in order to assess its potential to be used as a biosorbent.

Materials and Methods

In this study, four *Bacillus* sp., and two *Aspergillus niger* isolates were selected for biosorption of Cr^{6+}, which had been characterized earlier from soil contaminated with textile industry effluent (Faryal *et al*., 2007). Isolated bacterial colonies, after purification, were initially Gram stained and subsequently biochemically characterized and identified by using Bergey's Manual of Determinative Bacteriology (Kreig & Holt, 1984). For short term storage, bacteria were subcultured on nurtient agar slants and maintained at 4ºC, whereas, for long term storage, mid-exponential phase isolates were taken in nutrient broth, covered with 10% glycerol, and kept at –20ºC. Fungal strains were isolated and maintained on Sabouraud dextrose agar (SDA) slants at 4°C. During batch culturing in shake flasks, Sabouraud dextrose broth of the same composition except agar was used.

To determine the maximum resistivity levels (MRL), the method proposed by Price *et al.,* (2001) was used by adding Cr^{6+} salt ($K_2Cr_2O_7$). On the basis of MRLs shown by various bacterial and fungal isolates on metal amended agar plates, tolerant *Bacillus* sp., strains selected for shake flask experiments were *Bacillus* sp. RH69, *Bacillus* sp. RH70, *Bacillus pumilis* RH84 and *Bacillus fumus* RH109, alongwith fungal strains *Aspergillus niger* RH17 and *Aspergillus niger* RH19. All experiments were performed in duplicate to minimize errors in analysis.

Batch Cr^{6+} biosorption studies: Biosorption by *Bacillus* sp**.** was determined following the method proposed by Mclean & Beveridge (2001), by using minimal salt media, Vogel-Bonner (VB) broth, having glucose as the sole source of carbon. $K_2Cr_2O_7$ salt stock solution (96 mL) was added to 2mL VB broth and 2mL glucose (25%w/v) to attain 25 mg/L concentration of Cr^{6+}, and removal was studied after incubation at pH 5.0, 100 rpm agitation and 30^{o}C temperature for 3 days. Samples were drawn after every 24 hours and digested with 65% HNO_3 after filtration with 0.22μm milipore filter.

Biosorption by *Aspergillus niger* RH17 and *Aspergillus niger* RH19 was assessed by adding 25mg/L Cr^{6+} in 100 mL of SDB, inoculated with a spore suspension of $1.0x10^5$ spores/mL. Flasks were placed on an orbital shaker (100 rpm) at a temperature of 30°C and pH 5.0 for 3 days. Growth conditions of *Aspergillus niger* RH19 at which it had yielded the best growth on simple SDA, devoid of any metal ion, were also assessed for the further biosorption studies.

Effect of various conditions on biosorption: Initial effect of metal concentration on biosorption by *Aspergillus niger* RH19 was determined by adding different concentrations of metal ion, Cr^{6+}, 25-75mg/L, at 30°C, pH 5.0 and agitation of 100 rpm. *Aspergillus niger* RH19 was further studied for Cr^{6+}removal by determining the effect of pH (5.0- 9.0) and temperatures (25°, 30° and 35°C) in 100 mL of SDB containing 25 mg/L.

Samples drawn each day were filtered by Whatmann filter paper no. 1. Biomass was dried at 55°C in an oven till a constant weight was reached. After drying, it was weighed to determine the effect of different cultural conditions on the growth of fungal strains. Metal content was analyzed after digestion with 0.5 ml of 65% HNO_3 and heating in a water bath at 100°C for 5-6 hours.

Analytical methods: Metal ion concentrations, for determining metal removal both by bacterial and fungal strains, were analyzed on Air-Acetylene Varian – AA240FS Fast Sequential A4 Atomic Absorption Spectrophotometer, at 357.9 nm wavelength, with determination limit of 0.02 mg/L, sensitivity 0.1 mg/L and an optimum range of 0.2-10.00 ng/mL (Anon., 1992).

Bioaccumulation of Cr by *Aspergillus niger* RH 19 using biofermenter: On the basis of high metal removal capacity and ease to separate fungal biomass, *Aspergillus niger* RH19 was assessed for Cr^{6+} removal in a biofermenter. Metal uptake experiments were carried out using New Brunswick Scientific Fermentor/ Bioreactor flask of 1000 mL capacity, with a working volume of 600 mL. Growth media pH was adjusted to 6.0, temperature 35°C, agitation 100 rpm and 5% DOT (dissolved oxygen tension) for 15 days. pH was maintained to the desired value by 0.1N NaOH and 0.1N HCl. Cr^{6+} (25mg/L) was used for biosorption experiments over the period of 15 days. Samples were drawn every 3rd day. The metal ion concentration absorbed onto the biomass was calculated from the difference between metal concentration before and after the biosorption process.

Results and Discussion

To assess the tolerance of the two fungal strains against Cr^{6+}, *Aspergillus niger* RH17 and *Aspergillus niger* RH19 were incubated on Cr^{6+} amended plates. Among these two strains, *Aspergillus niger* RH19 showed maximum growth up to 275mg/L. Within 3 days of incubation, *A. niger* RH19 spread on the entire Petri plate, bearing spores between Cr^{6+} concentration range of 25 to 100 mg/L. From 125 to 150mg/L, it spread on the whole plate in 7 days, but without any spores. Whereas, at Cr^{6+} concentration range of 175 to 225 mg/L, the strain showed moderate growth even on the 7th day of incubation, while no growth was observed at 300mg/L concentration. On the other hand, *A. niger* RH17 showed no growth above 175 mg/L. Similar results have been reported for strains

of *Aspergillus flavus* and *Aspergillus niger* associated with marine seaweed (*Eucheuma* sp.), when tested for their Cr^{6+} tolerance, as both isolates had shown a luxuriant growth in different concentrations of Cr^{6+}, (25, 50 and 100 mg/L), which indicated that the isolates tolerated a wide range of hexavalent chromium and their application for bioremediation purpose could be envisaged (Vala *et al.,* 2004). Price *et al.,* (2001) tested different fungi by growing them on 5 mM Zn, and found that all the fungi tested were able to grow, however, *A. niger* grew better and obtained a colony diameter of 84.5 mm in 7 days.

Bacillus sp., RH69, *Bacillus* sp., RH70, *Bacillus pumilis* RH84 and *Bacillus fumus* RH109 showed maximum tolerance (mg/L), against Cr^{6+} in nutrient agar, which was 800, 900, 800 and 1000, respectively. Such a high level of metal resistance by bacteria has been reported previously (Megharaj *et al*., 2003). All strains used in this study showed very high-level resistance against potassium chromate both in nutrient agar and VB agar. Chromium resistant *Distigma proteus* isolated from effluent reportedly resist up to 12 µg/mL of Cr^{6+} in the medium (Rehman *et al*., 2007). Similarly chromium resistant bacteria isolated from tannery effluent have resisted 250 µg/mL of Cr^{6+} (Basu *et al*., 1997). Takeuchi *et al*., (2007) also reported strains, isolated from polluted marine environment, which could resist high levels of arsenate. Therefore, for the present study, *Bacillus* sp., RH69 and *Bacillus fumus* RH109 were selected for determining metal removal. Metal resistant microbes are prevalent in the polluted environment either due to development of resistance mechanisms, or already present inherent resistance attained through the evolutionary process.

Bacillus sp., RH 69 showed higher Cr^{6+} removal (42.60%) as compared to *Bacillus fumus* RH109 (38.85%), in the shake flask within 24 hours, after which desorption occurred, due to adsorption of metal ions onto the cell wall surface's charged groups which bind to these cations, and since this is a metabolism independent mechanism, it is a rapid process. *Aspergillus niger* RH19 removed 46.00% Cr^{6+} over the period of 3 days. Zhou *et al.,* (2007) also reported batch system Cr biosorption by dead biomass of *Bacillus licheniformis*, but at a higher temperature of 50°C, with pH 2.5 and 300 mg/g metal concentration. Since the aim of this work was to develop a low cost biosorbent which can grow on the industrial site and remove the metal *on site, Aspergillus niger* RH19 was selected for further shake flask experimentation due to the reason that it showed a high metal removal as compared to *Bacillus* sp., RH69, which removed the metal in a relatively short period, but in next few hours it was desorbed back to the media (Table 1). Another reason for selection of the fungal isolate for further studies was that it is cheap to grow the fungus and easy to remove it from the waste water after the treatment. The mechanisms associated with the biosorption of heavy metal ions using microorganisms as biosorbents are affected by several factors, which include the specific surface properties of the organism and the physiochemical parameters of the solution, such as, temperature, pH, initial metal ion concentration, and biomass concentration. Therefore, these factors were analyzed for Cr removal by *Aspergillus niger* RH19 before scaling up the process in a biofermentor.

Effect of metal concentration on Cr removal: Cr in aqueous form ($K_2Cr_2O_7$), representing different initial concentrations of Cr^{6+}, 25mg/L, 50mg/L and 75mg/L was added in the growth media. Temperature was kept at 30°C and pH was maintained at 5.0, which were the conditions at which best growth of the fungus was recorded in SDA, without any metal ion. Maximum biosorption of 75.35% was obtained at 25 mg/L on the 6^{th} day, which was reduced to 33.31% on the 15^{th} day. At 50 mg/L 45.49% removal was

Table 1. Percentage Cr^{6+} removal by *Bacillus* sp., RH69, *Bacillus fumus* RH109 and *Aspergillus niger* RH19 at 30°C, pH 5.0.

Days	*Aspergillus niger* RH19	*Bacillus* sp. RH69	*Bacillus fumus* RH109
Initial	0.00	0.00	0.00
Upon Inoculation	3.93	28.60	25.80
1	9.12	42.60	38.85
2	28.90	34.10	25.35
3	46.00	30.20	22.80

Table 2. Percentage removal of Cr^{6+} by *Aspergillus niger* RH19 from SDB, at 100 rpm agitation under different conditions.

Variable		Constants	Percentage metal removal						
Parameter	Level		Initial	Upon inoculation	3rd Day	6th Day	9th Day	12th Day	15th Day
Cr^{6+}	25 mg/L	30°C, pH 5.0	0.00	2.21	43.12	75.35	63.19	55.86	33.31
	50 mg/L		0.00	4.21	35.21	45.49	28.56	19.29	25.96
	75 mg/L		0.00	1.02	19.61	22.23	17.26	31.34	13.75
Temperature	25°C	25 mg/L, pH 5.0	0.00	0.59	23.20	19.20	16.80	14.90	12.80
	30°C		0.00	9.61	46.40	20.40	28.40	25.20	4.10
	35°C		0.00	3.28	46.00	23.60	11.20	37.00	24.80
‘pH	5.0	25 mg/L, 30°C	0.00	3.93	46.00	23.60	11.20	37.00	24.48
	6.0		0.00	4.98	35.20	45.20	60.80	60.00	50.00
	7.0		0.00	5.46	32.00	52.80	61.60	60.60	64.40
	8.0		0.00	4.32	46.80	56.00	54.40	65.00	74.00
	9.0		0.00	3.26	57.60	46.40	40.40	45.60	53.60

observed on the 6th day, while at 75 mg/L maximum 31.34% metal removal was recorded, but removal time was prolonged to the 12th day, which was followed by desorption (Table 2). Hence the increase in the initial concentration of the metal ion led to decrease in the removal of Cr^{6+}, indicating an effect of limited binding sites for metal ions available on the fungus, such as carboxylic, hydroxyl and phosphate group of lipids, proteins and polysaccharides localized at the cell surface for adsorption (Preetha & Viruthagiri, 2005). Contrary to our findings, increased chromate removal with increase in initial concentration has been documented earlier (Faisal & Hasnain, 2004; Rehman *et al*., 2007), while Vala *et al.,* (2004) reported low removal (25%) of hexavalent Cr by *Aspergillus flavus* and *Aspergillus niger*, with a change in the metal ion concentration.

Effect of temperature on Cr removal: During the optimization of temperature, the fungal strain was grown at three different temperatures, 25°C, 30°C and 35°C, with pH 5.0 and 25 mg/L metal concentration. At 25°C, 23.20% Cr^{6+} was removed on the 3rd day, whereas at 30°C and 35°C maximum removal was 46.40% and 46.00% respectively, also on the third day (Table 2). Although optimal growth temperature for *A. niger* RH19 was 30°C, but it also showed good removal at 35°C. Similar findings were observed by Nasseri *et al.,* (2002), where maximum biomass growth of *Aspergillus oryzae* and Cr removal rate was achieved at 30°C, and they reported decreasing temperature below 24°C decreased the fungal growth and enzymatic activity, while increasing the temperature up to 40°C decreased the fungal growth to minimal and consequently the Cr removal was negligible. Therefore, temperatures lower than 25°C and higher than 35°C were not included in our study. An earlier study conducted by Visoottiviseth & Panviroj (2001), reported that the best removal of arsenate by *Penecillium* sp. RRMT2-40l was observed at 27°C, whereas at 37°C no arsenate was removed. High temperature has been suggested to affect the integrity of cell membranes, and hinder compartmentalization of metal ions leading to reduced metal uptake.

Effect of pH on Cr removal: *Aspergillus niger* RH19 at different pH values (5.0-9.0), adjusted with HCl or NaOH, in shake flask experiments in SDB containing 25mg/L for each pH, were incubated for 15 days at 30°C. Better results for Cr^{6+} removal were recorded at pH 6.0 and 7.0, maximum removals being 60.80% and 61.60% on the 9th day, respectively. At pH 5.0, maximum removal of 46.00% was observed on day 3rd day. As the pH increased from 5.0 to 8.0, metal removal increased, but time to attain this also increased, as 56.00% Cr^{6+} was removed by *Aspergillus niger* RH 19 on the 6th day and 74.00% on the 15th day at pH 8.0 (Table 2). The uptake of metal ions by fungi is greatly affected by pH. Removal of Cr^{6+} varied with alteration in pH, with live and growing *Aspergillus niger* RH19 in the present study. Parvathi *et al*., (2007) also reported increase in manganese removal with rise of pH by *Aspergillus niger*. Diniz & Volesky (2005), suggested that metal binding increased with pH due to the decrease of proton concentration in the system, as they also compete for the binding sites.

The different pH binding profiles for heavy metal ions could be attributed to the nature of the chemical interactions of each metal with microbial cells and are related to the isoelectric point of the cell. At pH values above the isoelectric point, there is a net negative charge on the cells and the ionic state of ligands (carboxyl, phosphate, and amino groups) promotes reaction with the metal cations. As the pH is lowered, however, the overall surface charge on the cells becomes positive, which results in the inhibition of the approach of positively charged metal cations (Sağ & Kutsal, 1995). *Aspergillus niger* RH 19 removed Cr^{6+}under both acidic and alkaline condition similar to biotransformation of Hg(II) by cyanobacteria, over a wide range of pH (Lefebvre *et al.*, 2007).

Table 3. Biosorption of Cr^{6+} using stirred tank biofermentor by growing biomass of *Aspergillus niger* RH19.

Time since inoculation	Percentage removal
Initial	0.00
Upon Inoculation	3.21
3rd day	10.80
6th day	29.20
9th day	42.00
12th day	58.00
15th day	60.00

Bioaccumulation of Cr using stirred tank biofermentor: In the study under discussion, a stirred tank biofermentor was used to determine bioaccumulation capacity of *Aspergillus niger* RH19 for uptake of Cr^{6+}, which was comparable to shake flask. Maximum removal was observed to be 60% on 15th day (Table 3). Chirwa & Wang (2005) found improved Cr^{6+} removal using biofilm in a fixed bed reactor. Although in the shake flask *Aspergillus niger* RH19 removed more Cr^{6+} at pH 8.0, but pH 6.0 was selected for biofermentor studies as it was more close to its optimal growth pH. From this pilot study, it could be concluded that this fungus is a good candidate to scale up its production as a biosorbent, as it can grow and remove Cr^{6+} over a wide range of pH and temperature. However, further studies are needed to optimize the conditions for *onsite* metal removal.

Conclusion

The preliminary study presented here shows that *A. niger* RH19 biomass is an effective absorbent for the removal of heavy metals, specially Cr^{6+}, from assimilated waste waters, from amongst the various microbial strains assessed. It exhibited good biosorption at 30°-35°C, and pH had a profound effect on biosorption, as the pH was increased the metal removal also increased, with the effective pH range being 6.0-8.0. *A. niger* RH19 showed adsorption as well bioaccumulation of Cr^{6+}, which is indicated by metal removal as soon the inoculum is added to media in the shake flask studies. As we scaled up the experiment from 100 mL to 600 mL in the fermentor, increase in volume did not effect the percentage removal of the metal. Further work is needed to develop it into a low cost biosorbent, for application in the small tannery industries of Pakistan.

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