Chromium removal from a real tanning effluent by autochthonous and allochthonous fungi

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Valeria Prigione, Mirco Zerlottin, Daniele Refosco, Valeria Tigini, Antonella Anastasi, Giovanna Cristina Varese

Abstract

Heavy metals represent an important ecological and health hazard due to their toxic effects and their accumulation throughout the food chain. Conventional techniques commonly applied to recover chromium from tanning wastewaters have several disadvantages whereas biosorption has good metal removal performance from large volume of effluents. To date most studies about chromium biosorption have been performed on simulated effluents bypassing the problems due to organic or inorganic ligands present in real industrial wastewaters that may sequestrate the Cr(III) ions. In the present study a tanning effluent was characterized from a mycological point of view and different fungal biomasses were tested for the removal of Cr(III) from the same tanning effluent in which, after the conventional treatments, Cr(III) amount was very low but not enough to guarantee the good quality of the receptor water river. The experiments gave rise to promising results with a percentage of removed Cr(III) up to 40%. Moreover, to elucidate the mechanisms involved in biosorption process, the same biomasses were tested for Cr(III) removal from synthetic aqueous solutions at different Cr(III) concentrations.

1. Introduction

Increasing industrial development and urbanisation has resulted in generation of large quantities of toxic substances, introduced into the environment with risk for living organisms, and potentially constituting serious hazards to public health. The presence of toxic heavy metal contaminants in aqueous streams, arising from dumping of metal containing effluents into water bodies, is one of the most important environmental issues (Melgar et al., 2007 and Kumar et al., 2008).

Chromium is naturally found in rocks, soil, plants, animals, volcanic dust and gases and exists primarily as the soluble, highly toxic Cr(VI) anions and the less soluble and less toxic Cr(III) (Kumar et al., 2008), which is the most stable valence state of chromium, in aqueous media, at pH values between 4 and 10 (Calfa and Torem, 2008). Even if Cr(III) is an essential element, it can be toxic at elevated concentrations in the environment (Calfa and Torem, 2008). Chromium has widespread industrial applications, such as tanning industries, electroplating, textile dying, wood preservation, as well as finishing of metals and plastics. As a result of these applications, chromium enters in the effluent streams, thereby affecting the environment adversely (Agrawal et al., 2006). Conventional methods for removing metals from aqueous solutions include chemical, physical methods (chemical precipitation, chemical oxidation or reduction, ion exchange, filtration, electrochemical treatment, reverse osmosis, membrane technologies, evaporation recovery, etc.) and activated sludge biological treatment (Ahluwalia and Goyal, 2007). These processes are generally efficient in removing the bulk of metal from solution at high or moderate concentrations, whereas they may be ineffective or extremely expensive especially when the metals in solution are at low concentration i.e. in the range of 1–100 mg l^{-1} (Ahluwalia and Goyal, 2007). As a consequence, their limits (high cost, high reagent requirements, etc.) become more pronounced when voluminous effluents containing complexing organic matter and low metal contamination must be treated. Biotechnological approaches can succeed in those areas and are designed to cover such niches (Malik, 2004).

Among biotechnological techniques, biosorption is viewed as one of the most valuable choices for the removal of heavy metals from wastewaters. Recently, the discovery of biosorbents, which exhibit a favourable cost-efficiency relationship, when compared to conventional sorbents (i.e. activated carbon and ion exchange resins), has been proved to be a reality (Calfa and Torem, 2008). Microorganisms

including bacteria, filamentous fungi and yeasts are found to be capable of efficiently accumulating heavy metals. The ability of fungi to act as biosorbents has been extensively evaluated and they have shown excellent metal sequestering abilities for heavy metals such as Cd, Cu, Zn, Pb, Fe, Ni, Ag, Th, Ra and U from aqueous solution (Ahluwalia and Goyal, 2007, Kapoor and Viraraghavan, 1995 and Mungasavalli et al., 2007). Biosorption encompasses a number of metabolism-independent processes (physical and chemical adsorption, electrostatic interaction, ion exchange, complexation, chelation and micro precipitation) that mainly take place at the cell wall level. Both living and dead fungal biomasses can be used to remove metals, but dead cells are obviously preferable for wastewater treatment since they are not affected by toxic wastes and chemicals and do not pollute the environment by releasing toxins and/or propagules. Besides, dead and dried biomasses can be stored for long periods at room temperature with little risk of putrefaction, making them easier to use and transport. Moreover, dead fungal biomasses can be available in substantial quantities from various industrial fermentation processes, which could serve as an economical and constant supply source of biomass for the removal of metal ions (Kumar et al., 2008,Prigione et al., 2008a and Prigione et al., 2008b). Finally, dead biomasses can be subjected to physical and chemical treatments to enhance their performances (Kapoor et al., 1999).

In the recent past, biosorption studies involving different kinds of dead or live biomasses have dominated the literature. Metal uptake capacity by various biosorbents has been evaluated using biosorption isotherm curves derived from equilibrium batch sorption experiments. Effects of various process parameters such as pH, biomass loading, biomass pre-treatments, etc., have been studied extensively (Malik, 2004, Sag et al., 2000 and Yu and Kaewsarn, 2000). Although high biosorptive potential for several types of microbial biomass has been reported, their value under real conditions remains to be tested because most of the metal biosorption studies are conducted using synthetic metal solutions and not real industrial effluents (Malik, 2004).

In the present study a tanning effluent was characterized from a mycological point of view. Two fungal strains (*Fusarium solani* and *Mucor circinelloides*) isolated from the effluent and 3 strains (*Cunninghamella elegans*, *Rhizomucor pusillus* and *Rhizopus stolonifer*) previously studied for dye biosorption were investigated, by batch experiments, for Cr(III) removal from the same tanning

effluent, in which Cr(III) amounts, after the chemical, physical and biological conventional treatments, was very low but not enough to guarantee the good quality of the receptor water river, being above the threshold limit value (0.25 mg l^{-1}) of the Regione Veneto general policy law. Fungal biomasses were inactivated and then physically (lyophilisation) or chemically (boiling in NaOH) pre-treated in attempt to improve their efficiencies in Cr(III) removal. In order to elucidate the mechanisms involved in biosorption, the same biomasses were tested for Cr(III) removal from synthetic aqueous solutions at different Cr(III) concentrations.

2. Methods

2.1. Raw and chemically treated tanning effluent and preparation of Cr(III) synthetic solutions

Samples of effluent were collected after the chemical, physical and biological conventional treatments, at Acque del Chiampo S.p.a. Servizio Idrico Integrato plant (Arzignano, Italy), which treats about 30.000 m^3 day^{-1} of effluent originating from 160 tanning industries. The Cr(III) content of the effluent samples used for the experiments was in the range of 0.31–0.56 mg l^{-1}, while Cr(VI) was always absent. The initial pH of the effluent samples used ranged from 7.80 to 8.20; it was not maintained constant during the experiments using a buffer. The physical–chemical properties of the effluent are listed in Table 1.

Table 1
Characteristics of the tanning wastewaters after the chemical, physical and biological conventional treatments (mean values of one year monitoring).

Parameters	Characteristics
pH	8.15
COD	155 mg l^{-1}
BOD_5	11 mg l^{-1}
TKN	10 mg l^{-1}
$N–NH_4$	0.6 mg l^{-1}
$N–NO_3$	10 mg l^{-1}
Total suspended solids	17 mg l^{-1}
Cloride	2340 mg l^{-1}
Sulphate	2080 mg l^{-1}
Fe	0.14 mg l^{-1}
Cr(III)	0.45 mg l^{-1}

In some trials, part of the tanning effluent was treated by clariflocculation with inorganic salts which was performed adding 25 mg l^{-1} of 18% aluminium polychloride solution in Al_2O_3 and 35 mg l^{-1} of 40% ferric chloride solution (Unichimica, Torri di Quartesolo, Italy), and stirring for 10 min. Then 1 mg l^{-1} of anionic polyelectrolyte, copolymer of acrylamide/sodium acrylate (SNF Acque Italia, Nova Milanese, Italy), was added and finally, after the settling of the inorganic sludge, the supernatant was collected.

The Cr(III) synthetic solutions were generated by dissolving chromium nitrate nonahydrate, $Cr(NO_3)_3 \cdot 9H_2O$ (Sigma‐Aldrich, St. Louis, MO), in distilled water in order to obtain the following final concentrations of Cr(III): 0.4, 4, 40 and 400 mg l^{-1}. The initial pH of synthetic solutions was 5; it was not maintained constant during the experiment using a buffer.

2.2. Isolation of fungal strains from the tanning effluent

The two media used for fungal strains isolation were the generic MEA (20 g l^{-1} malt extract, 20 g l^{-1}glucose, 2 g l^{-1} peptone, 18 g l^{-1} agar) and the synthetic EQ-Cr (20 g l^{-1} glucose, 2 g l^{-1} ammonium tartrate, 2 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.1 g l^{-1} $CaCl_2 \cdot 2H_2O$, 3.8 g l^{-1} NaCl, 2.8 g l^{-1} Na_2SO_4, 5 mg l^{-1} $Cr(NO_3)_3 \cdot 9H_2O$, 10 ml mineral stock solution). The mineral solution contained, per 100 ml of distilled H_2O, 0.05 g $MnSO_4 \cdot 5H_2O$, 0.1 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $CoCl_2 \cdot 6H_2O$, 0.01 g $ZnSO_4 \cdot 7H_2O$, 0.001 g $CuSO_4 \cdot 5H_2O$, 0.001 g $AlK(SO_4)_2$, 0.001 g H_3BO_3, 0.001 g $NaMoO_4 \cdot 2H_2O$. The concentration of chlorides, sulphates and Cr(III) in EQ-Cr was comparable to those of the tanning effluent. Both media were supplemented with 15 mg l^{-1} streptomycin sulphate and 50 mg l^{-1} chloramphenicol to inhibit the bacteria growth.

Three ml of tanning effluent were spread on plates (50 of MEA and 50 of EQ-Cr), then incubated at 30 °C, temperature close to that of the effluent leaving the treatment plant. After about 3‐4 days, the plates were screened to identify and count the colonies; serial readings were made to isolate and track the progress of slowly growing fungi for 2 weeks. Fungi were identified conventionally according to their macroscopic and microscopic features. After determination of their genera, they were transferred to media recommended by the authors of selected genus monographs for species identification. Sterile mycelia were classified according to their hyphal pigments.

The significance of differences ($P \leqslant 0.05$) between the load of each fungal entity grown on MEA and EQ-Cr was calculated by the unpaired *t*-test (SYSTAT 10 for windows SPSS inc., 2000).

2.3. Test organisms

Fusarium solani (Mart.) Sacc. and *Mucor circinelloides* f. *griseocyanus* (Hagem) Schipper were isolated from the studied tanning effluent. *Cunninghamella elegans* Lendner (MUT 2861*), Rhizomucor pusillus*(Lindt) Schipper (MUT 2229) and *Rhizopus stolonifer* (Ehrenberg) Vuillemin (MUT 1515) were obtained from the *Mycotheca Universitatis Taurinensis* Collection (MUT, University of Turin, Department of Plant Biology). They are patented species for dye biosorption.

Starting cultures were lyophilised and cryopreserved until use. They were revitalised on MEA and mature conidia for the inocula and biomass production were obtained from cultures grown on the same medium in the dark at 24 $^{\circ}$ C for 1 week.

2.4. Fungal biomass preparation

The two media used for biomass production were EQ (20 g l^{-1} glucose, 2 g l^{-1} ammonium tartrate, 2 g l^{-1} l KH_2PO_4, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.1 g l^{-1} $CaCl_2 \cdot 2H_2O$, 10 ml mineral stock solution) and ST, in which 18 g l^{-1} potato starch is used instead of glucose (Fluka, St. Luis, MO). Since the literature (Ellis et al., 1974) and preliminary tests had shown that *R. stolonifer* cannot use starch as a carbon source, whereas *R. pusillus*, *M. circinelloides* and *F. solani* did not produce substantial amount of biomass on ST, these fungi were cultured on EQ only. Fungi were inoculated as a conidial suspension (final concentration of 2.5×10^5conidia ml^{-1}) in several 500 ml Erlenmeyer flasks containing 300 ml of medium, and incubated at 30 $^{\circ}$ C for 7 days. To avoid production of aerial mycelium, rich in hydrophobins and poorly adsorbent, biomasses were cultured in agitated condition at 110 rpm with a Minitron Infors orbital shaker (Bottmingen, CH). After incubation, the biomass was collected with a sieve (150 μm pore), homogenized, rinsed several times with distilled sterilised water to remove residual medium and then inactivated in saline solution by autoclaving at 121 $^{\circ}$ C for 30 min. It was then collected in sterile conditions and rinsed as already described.

2.5. Chemical and physical biomass pre-treatment

After the inactivation, part of the *C. elegans* biomass was lyophilised (Lyophiliser LIO 10P, Cinquepascal, Trezzano s/n, Italy) and then powdered to particles of uniform size (300 μ m < Ø < 600 μ m). Another part of the inactivated *C. elegans* biomass was boiled in 0.5 M NaOH solution for 15 min and then washed with generous amounts of deionised water as long as the pH of the washing solution was in the near-neutral range (7.0–7.2).

2.6. Batch biosorption experiments

Each biomass type was weighed and 15 g fresh weight, in the case of fresh biomasses, and 1.5 g dry weight of lyophilised one (corresponding to about 15 g of fungal biomass fresh weight) were placed in 250 ml Erlenmeyer flasks containing 150 ml of real or synthetic effluent. The flasks were incubated at 30 $^{\circ}$ C in agitated conditions (110 rpm). Samples of effluent without biomass were used as abiotic control. Each trial was performed in triplicate.

After 2, 6 and 24 h, or after 24 h only, the effluent was analysed (APAT CNR IRSA 3010 Man 29 2003 + APAT CNR IRSA 3020 Man 29 2003) in order to determine the percentage of Cr(III) removed (RP). At the end of the experiment the biomasses were filtered on filter paper (Whatman type 1), placed in an oven and dried at a temperature of 65 $^{\circ}$ C for 24 h; then they were weighed in order to calculate the sorption capacity (SC), a parameter that takes into consideration the maximum sorbent capability, according to the following formula:

SC=mg of Cr(III)removed/g of biomass dry weight

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The influence of the incubation time on the biosorption yield was assessed from the increase in Cr(III) removal from the 2nd to the 24th h, calculated as RP at T24 – RP at T2)· 100/ RP at T24.

The significance of differences ($P \leqslant 0.05$) among the RP values at 2, 6 and 24 h and among SC values was calculated with the Mann-Whitney test (SYSTAT 10 for windows SPSS inc., 2000).

3. Results

3.1. Isolation of fungal strains from the tanning effluents

Table 2 shows the fungal entities isolated on MEA and/or EQ-Cr and their load (CFU l^{-1} of effluent). The total fungal load isolated on MEA and EQ-Cr were 1820 and 1607 CFU l^{-1} of effluent respectively. A total of 25 genera of filamentous fungi and 2 groups of sterile mycelia, namely *mycelia sterilia moniliacea* and*dematiacea*, together with several yeasts were isolated. Very few colonies were not isolated (unidentified fungi group in Table 2) since they were early held by rapidly growing and abundantly sporulating invasive species. A total of 49 species were identified (36 on MEA, 32 on EQ-Cr) of which 19 (40.8%) were isolated on both media, 17 (34.7%) exclusively on MEA, 13 (26.5%) exclusively on EQ-Cr. *Phialocephala*,*Trichoderma* and *Penicillium* species were dominant on both media, accounting for 56% of the total load on MEA and 61% on EQ-Cr. No significant differences in the load of species isolated on both media were observed, with the exception of *Fusarium* sp., prevailing on MEA and *Penicillium waksmanii*, prevailing on EQ-Cr.

Table 2
Fungal entities isolated on MEA ed EQ-Cr and their load (mean of 50 replicates for each medium).

Fungal entities	Load (CFU l^{-1} of effluent)	
	MEA	EQ-Cr
Acremonium sp.	6.67	–
Aphanocladium album (Preuss) W. Gams	–	13.33
Aspergillus flavus Link var. *flavus*[1,2,7]	13.33	20.00
Aspergillus fumigatus Fresen. var. *fumigatus*[1,2]	–	6.67
Aspergillus niger Tiegh. var. *niger*[1],	–	33.33
Aspergillus oryzae (Ahlb.) E. Cohn	20.00	13.33
Aspergillus sp.	13.33	–
Aspergillus terreus Thom var. *terreus*	6.67	–
Aspergillus ustus (Bainier) Thom & Church	–	6.67
Byssochlamys nivea Westling var. *nivea*	6.67	–
Chrysosporium sp.	6.67	–
Epicoccum nigrum Link	6.67	–
Eupenicillium sp.[4]	–	6.67
Eurotium amstelodami L. Mangin	6.67	–
Exophiala sp.[2]	106.67	60.00
Fusarium solani (Mart.) Sacc.[1,2,3]	26.67	26.67
Fusarium sp.	93.33	26.67*
Fusarium subglutinans (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas	13.33	–
Geotrichum candidum Link[1]	46.67	73.33
Gliocladium sp.[1,3,6]	13.33	20.00
Monocillium sp.	40.00	60.00
Mucor circinelloides f. *griseocyanus* (Hagem) Schipper [1,2,3,4,6]	20.00	6.67
Mycelia sterilia dematiacea	20.00	–
Mycelia sterilia moniliacea	26.67	–
Paecilomyces lilacinus (Thom) Samson[2,6]	13.33	6.67
Paecilomyces variotii Bainier[1,2]	73.33	66.67
Penicillium citrinum Thom[1,2]	–	26.67
Penicillium decumbens Thom	13.33	–
Penicillium jensenii K.M. Zalessky	6.67	–
Penicillium lollense Pitt	6.67	–
Penicillium nalgiovense Laxa	–	6.67
Penicillium ochrochloron Biourge	53.33	–
Penicillium oxalicum Currie & Thom[5]	93.33	13.33
Penicillium pinilli Bainier	–	6.67
Penicillium sp.1	6.67	–
Penicillium sp.2	–	6.67
Penicillium sp.3	–	6.67
Penicillium spinulosum Thom	80.00	6.67
Penicillium variabile Sopp	6.67	–
Penicillium viridicatum Westling	–	6.67
Penicillium waksmanii K.M. Zalessky	13.33	193.33*
Phialocephala sp.[7]	553.33	440.00
Pleurostomophora richardsiae (Nannf.) L. Mostert, W. Gams & Crous[1]	6.67	6.67
Pseudallescheria boydii (Shear) McGinnis, A.A. Padhye & Ajello	60.00	73.33
Sporothrix sp.	113.33	–
Talaromyces sp.[6]	20.00	20.00
Trichocladium opacum (Corda) S. Hughes	–	6.67
Trichoderma citrinoviride Bissett	6.67	–
Trichoderma harzianum Rifai[1,6]	180.00	260.00
Unidentified ascomycetes	6.67	–
Unidentified fungi	6.67	13.33
Westerdykella sp.[6]	–	6.67
Yeasts	6.67	60.00
Number of fungal entities	36	32
Total load (CFU l^{-1} of effluent)	1820	1607

* Indicate significant differences between MEA and EQ-Cr for the same fungal entity.
[1–7] References in which that fungi have already been reported as components of the mycoflora of sediments, soils and rivers polluted by heavy metals and polycyclic aromatic hydrocarbons: (1) El-Morsy, 2004; (2) Götrlich et al., 2002; (3) Doggett, 2000; (4) Baudino, 2001; (5) Massaccesi et al., 2002; (6) da Silva et al., 2003; (7) Bois et al., 2006.

3.2. Cr(III) removal from the industrial effluent

The percentages of Cr(III) removal by the inactivated biomass of the 5 fungal species are set out in Table 3. The Cr(III) concentration in abiotic controls never varied during the tests. Among the strains cultured in EQ, the highest Cr(III) removal percentages were obtained by *C. elegans* (30%) and *R. pusillus* (28%). The biosorption yield was significantly improved culturing *C. elegans* biomass on ST (38%).

Table 3
Percentage of Cr(III) removal from the tanning wastewater after 24 h incubation by different kinds of inactivated biomass.

Species	Culture medium	Percentage of Cr(III) removal (mean ± st. dev.)
Fusarium solani	EQ	16.87 ± 1.38^{a}
Mucor circinelloides	EQ	18.80 ± 1.48^{ac}
Rhizomucor pusillus	EQ	27.93 ± 1.56^{b}
Rhizopus stolonifer	EQ	20.51 ± 0.00^{c}
Cunninghamella elegans	EQ	29.73 ± 0.00^{b}
	ST	37.61 ± 1.48^{d}

^{a,b,c,d} Indicate significant differences.

Table 4
Percentage of Cr(III) removal from the tanning wastewater after 2 h (T2), 6 h (T6) and 24 h (T24) incubation by pre-treated biomasses of *Cunninghamella elegans*, increase of Cr(III) removal from T2 to T24 and sorption capacity (mg of Cr(III) g^{-1} of biomass dry weight).

Initial Cr(III) concentration (mg l^{-1})	Biomass state	Percentage of Cr(III) removal (mean ± st. dev.)			Increase of Cr(III) removal from T2 to T24 (%)	Sorption capacity (mean ± st. dev.)
		T2	T6	T24		
0.39 ± 0.00	Untreated	22.22 ± 1.48^{A}	27.35 ± 1.48^{B}	37.61 ± 1.48^{C}	40.9	0.012 ± 0.002
	Lyophilised	15.31 ± 3.26^{A}	22.91 ± 4.98^{AB}	28.34 ± 0.00^{B}	45.9	0.009 ± 0.000*
0.42 ± 0.02	Untreated	12.07 ± 1.38^{A}	16.87 ± 1.38^{B}	26.46 ± 1.38^{C}	54.4	0.016 ± 0.003
	NaOH pre-treated	9.67 ± 1.38^{A}	20.06 ± 1.38^{B}	32.05 ± 1.38^{C}	69.8	0.030 ± 0.002*

^{A,B,C} Indicate significant differences among sorption capacities at T2, T6 and T24 for the same biomass.
* Indicate significant differences among sorption capacity of untreated and pre-treated biomasses for the same effluent.

The percentages of Cr(III) removal by clariflocculation, biosorption by *C. elegans* biomass and the combination of the two treatments are shown in Fig. 1. The clariflocculation reduced the Cr(III) content in the effluent from 0.44 to 0.37 mg l^{-1} (about 16%); whereas about 40% of Cr(III) was removed within 24 h (more than 23% within 2 h) by the biosorption treatment. The total Cr(III) removal obtained by

the combined effect of clariflocculation and biosorption was about 44% and allowed to reduce the Cr(III) concentration in the effluent to less than 0.25 mg l^{-1}.

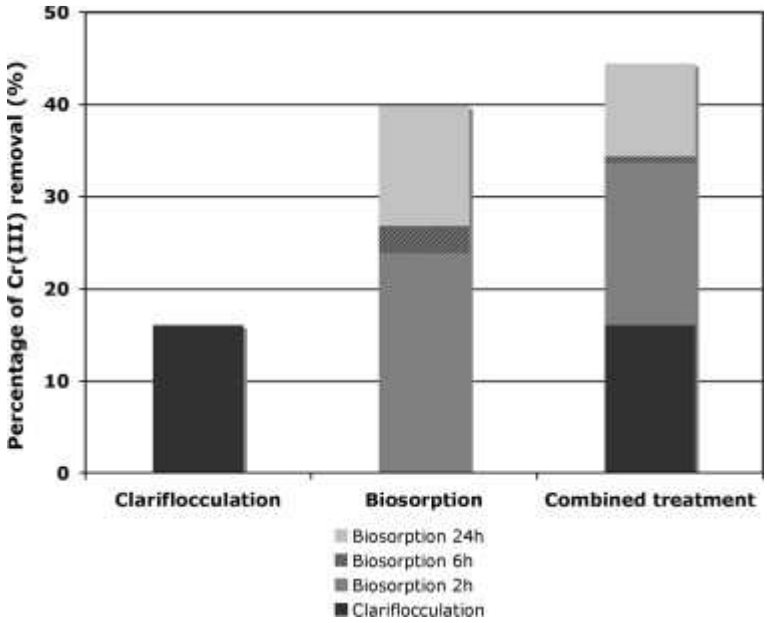

Fig. 1. Percentage of Cr(III) removal by clariflocculation, biosorption (after 2 h, 6 h and 24 h incubation with *Cunninghamella elegans*biomass) and by the combination of the two treatments, towards a real tanning effluent containing 0.44 mg l^{-1} Cr(III).

3.3. Cr(III) removal from synthetic aqueous solutions

The percentages of Cr(III) removal and the SC values of the inactivated and NaOH pre-treated biomasses of *C. elegans* towards synthetic solutions at different Cr(III) concentrations are set out in Table 5. The Cr(III) concentration in abiotic controls never varied during the tests. The NaOH pre-treated biomass always displayed significant higher SC values respect to the untreated one irrespective of the metal concentration. Moreover, both for the untreated biomass and the pre-treated one, the SC significantly increased as the initial Cr(III) concentration increased; the highest SC value (16 mg g^{-1}) was obtained by the NaOH pre-treated biomass towards the more concentrated synthetic solution.

Table 5
Percentages of Cr(III) removal after 2, 6 and 24 h incubation in synthetic aqueous solutions at different Cr(III) concentrations by the inactivated biomass of *Cunninghamella elegans*, increase of Cr(III) removal from T2 to T24 and sorption capacities (mg of Cr(III) g^{-1} of biomass dry weight).

Initial Cr(III) concentration(mg l^{-1})	Biomass state	Percentage of Cr(III) removal (mean ± st. dev.)			Increase of Cr(III) removal from T2 to T24 (%)	Sorption capacity (mean ± st. dev.)
		T2	T6	T24		
0.4	Untreated	11.36 ± 0.00^{A}	11.36 ± 0.00^{A}	11.36 ± 0.00^{A}	-	0.005 ± 0.000
	NaOH pre-treated	48.48 ± 4.28^{A}	56.06 ± 2.14^{B}	72.73 ± 0.00^{C}	33.3	$0.063 \pm 0.002^{*}$
4	Untreated	13.64 ± 0.00^{A}	13.64 ± 0.00^{A}	14.77 ± 1.61^{A}	7.7	0.066 ± 0.008
	NaOH pre-treated	38.10 ± 6.734^{A}	30.16 ± 0.00^{B}	89.52 ± 0.00^{C}	57.4	$0.745 \pm 0.003^{*}$
40	Untreated	9.524 ± 0.00^{A}	9.52 ± 0.00^{A}	11.90 ± 0.00^{B}	20.0	0.515 ± 0.008
	NaOH pre-treated	83.85 ± 2.38^{A}	88.03 ± 9.71^{A}	93.49 ± 0.34^{B}	10.3	$9.898 \pm 0.251^{*}$
400	Untreated	6.93 ± 0.69^{A}	7.65 ± 0.00^{A}	8.87 ± 1.37^{A}	21.9	3.960 ± 0.429
	NaOH pre-treated	18.20 ± 0.17^{A}	15.45 ± 1.02^{B}	16.17 ± 0.68^{B}	-	$15.941 \pm 1.368^{*}$

^{A,B,C} Indicate significant differences among sorption capacities at T2, T6 and T24 for the same biomass.
* Indicate significant differences among sorption capacity of untreated and pre-treated biomasses for the same effluent.

Lyophilised biomass was absolutely ineffective against simulated effluents (data not shown), since it was unable to remove the Cr(III) ions at all the tested concentrations.

A pH decrease, directly proportional to the amount of Cr(III) removed was observed. This linear relationship was particularly evident in the case of NaOH pre-treated biomass (R^2 = 0.933).

4. Discussion

A prolonged exposure to heavy metals exerts a highly selective pressure on the fungal community, which could lead to the appearance of metal resistant strains. Fungi can develop a high resistance to heavy metals by a variety of mechanisms to remove ions, such as adsorption to cell surfaces, complexation by exopolysaccharides, intracellular accumulation or precipitation (Massaccesi et al., 2002 and Saxena and Bhattacharyya, 2006). Hence, isolating fungi from polluted environments would represent an appropriate practice to select metal resistant strains that could be used for heavy metal removal and bioremediation purposes (Malik, 2004 and Zucconi et al., 2003).

The values of total load registered both for MEA and EQ-Cr (1820 and 1607 CFU l^{-1} of effluent respectively) are included in the range (as close to the lower limits)

obtained by other authors in studies on the mycoflora of surface water and groundwater for potability. In such environments, in fact, fungi are counted on average from 1000 to 10,000 CFU l^{-1}, with peaks that can reach 30,000 CFU l^{-1} (Göttlich et al., 2002). However, in our case, rather low values of total load correspond to a high biodiversity, as number of isolated species. This is apparently in contrast to what is generally observed for polluted environments, since the primary effect of the presence of toxic substances is the reduction of the number of species. A possible explanation for this phenomenon may be the low toxicity of the effluent due to the poor Cr(III) concentration.

Several species isolated on EQ-Cr have been reported in literature as components of the mycoflora of sediments, soils and rivers polluted by heavy metals and polycyclic aromatic hydrocarbons (Table 2), whereas the species isolated only on MEA, mostly with low load, are likely to be air pollutants, accidentally deposited in the effluent as propagules.

Among the fungi isolated from the tanning effluent on EQ-Cr, *F. solani* was selected for biosorption experiments since it was the only fungus able to grow *in vitro* into the tanning effluent, demonstrating a real adaptation to this polluted environment (data not shown). This characteristic made it potentially useful both for Cr(III) active uptake and for biosorption in the inactivate state, which are the two main strategies for the bioremediation of effluents polluted by heavy metals. Even if we decided to initially focus on the use of dead biomasses, for obvious applicative advantages, in the future it would be very interesting to assess the Cr(III) active uptake capacity of this fungus. Besides, some species belonging to the genus *Fusarium* have already been studied by other authors for their potential in biosorption of heavy metals and dyes (Delgado et al., 1998, Sen et al., 2005 and Zeroual et al., 2006). *Mucor circinelloides* was selected according to literature data since species belonging to Zygomycetes are reported to be characterized by special heavy metals biosorbent properties (Ahluwalia and Goyal, 2007, Bai and Abraham, 2002, Kapoor and Viraraghavan, 1995, Tobin and Roux, 1998 and Yan and Viraraghavan, 2003).

Many sea weeds, bacteria, yeasts and filamentous fungi have already been investigated for metal-binding capacities and fungi seem among the most promising since their cell wall surface contains many functional groups of carboxyl, hydroxyl, sulphidryl, amino groups and phosphate group of lipids, proteins and polysaccharides having ability to bind metal ions (Deng and Ting, 2005, Kumar et al.,

2008 and Sag, 2001). However, most of these studies have been performed using synthetic metal solutions.

This research demonstrates for the first time the potential of the Zygomycetes *C. elegans* to remove the Cr(III) ions from a real industrial tanning effluent. Among the 5 tested fungal species, the *C. elegans*biomass cultured in ST medium displayed the highest SC value. This fungus had never been previously employed for the removal of Cr(III) from industrial tanning effluents. However, its biosorption capabilities against textile dyes are well known (Prigione et al., 2008a and Prigione et al., 2008b) and chitin and chitosan extracted from *C. elegans* mycelium has been evaluated, with good results, for copper, lead and iron biosorption in aqueous solution (de Oliveir a Franco et al., 2004).

The influence of the growing medium (EQ *vs* ST) on *C. elegans* biomass was considered. On several occasions it has been reported that the cell wall responds to the culture medium and other properties of the environment by greatly changing its composition and chemical‐physical properties (Nemcovic and Farkas, 2001 and Znidarsic et al., 1999). Moreover, the C:N ratio in the medium can affect the amount of structural compounds (chitin‐chitosan) and other chemical groups of the cell wall (Znidarsic et al., 1999). In our case the *C. elegans* biomass cultured on the medium containing starch as carbon source displayed a higher SC value than that cultured on the medium containing glucose. This result is of great significance for the application of this biosorption method in industry since starch is a low-cost by-product of many manufacturing processes whose use as a source of carbon would reduce the generally very expensive production of biomasses. For these reasons, the following experiments were carried out using the *C. elegans* biomass cultured in ST only.

Chemical and physical pre-treatments often increase the biosorption capacity of fungal biomass by means of different mechanisms: (i) the removal of the outer cell wall layer (Gallagher et al., 1997 and Fu and Viraraghavan, 2002); (ii) the loss of cell integrity with a consequent increase of the surface area (Polman and Breckenridge, 1996); (iii) the increased porosity of cell wall with a higher exposure of its latent sites (Gallagher et al., 1997 and Polman and Breckenridge, 1996); (iv) the increased ratio between the cell wall and total biomass, due to the partial loss of hyphal lumen components (e.g. cytoplasm and organelles). In particular, previous studies on biosorption of different metal cations, such as Cu(II), Pb(II), Cd(II) by fungal

biomasses have demonstrated that NaOH pre-treatment could substantially increase the biosorption yields (Kapoor et al., 1999). Our results show that this chemical pre-treatment significantly enhanced the biomass SC also for Cr(III) both from tanning wastewater and synthetic aqueous solution. Such an improvement could be due to the exposure of active metal-binding sites embedded in the cell wall causing the availability of more anionic sites (Dursun, 2006). Actually, it must be considered that the treatment with cold alkali could extract important components of the fungal cell wall, mainly several types of α- and β-glucans, revealing the functional groups of other components such as the alkali-insoluble chitin and chitosan (Ruiz-Herrera, 1992). The lyophilisation process, on the contrary, did not improve the biosorption yield, nevertheless it could be considered a good way for biomass preservation and management.

The biomass pre-treatments also influenced the rapidity of the biosorption process. In particular, it is interesting to note that at a contact time of two hours the untreated biomass removed a higher amount of Cr(III) than the physically and chemically pre-treated biomasses. In fact, preliminary analysis with the Fourier transform infrared spectroscopy (FTIR, data not shown) on the different kinds of biomass showed that the NaOH treatment caused substantial changes, in terms of structure and composition of the cell wall, affecting both the type of binding between Cr(III) ions and cell wall functional groups, and the biosorption rate. On the other hand, the FTIR analysis showed that lyophilisation did not cause substantial changes in the cell wall composition. However, the lyophilised biomass had a lower biosorption yield especially after two hours of incubation. This delay could be explained by the fact that the lyophilised biomass needs a certain period of time to re-hydrate before starting the biosorption process.

Particularly interesting from an applicative point of view is the comparison between the results obtained by biosorption and clariflocculation. Biosorption resulted more effective than clariflocculation in Cr(III) removal; moreover by combining the two process, a total removal of Cr (III) of 44% was obtained, allowing to reduce the Cr(III) concentration below the threshold limit value (0.25 mg l^{-1}) of the Regione Veneto general policy law. This suggests that biomass-based technologies need not necessarily replace the conventional treatment routes but may complement them. Actually, a single universally applicable end-of-pipe solution appears to be unrealistic, and combination of appropriate techniques is deemed imperative to devise technically and economically feasible options (Hai et al., 2007).

Our SC values obtained with the real tanning wastewater are lower than that obtained by Tobin and collaborators (1984) for Cr(III) biosorption by the fungus *R. arrhizus* (31 mg g^{-1}) and, in general, than those reported in literature for other metal species (Kapoor and Viraraghavan, 1995 and Sag, 2001), but in our opinion such a comparison does not hold since most literature data refer to experiments carried out using synthetic metal solutions composed by metal salts dissolved in water at high concentration, so that the metal ions are ready available to the biomass. Moreover, in presence of a low concentration of metal ions as in the real effluent, there is a weak driving force of the pollutant in solution which results in a slow biosorption process.

The use of real tanning effluents for biosorption experiments poses several problems, mainly due to the low metal content and to the presence of organic or inorganic complexing matter that sequestrates metal ions. Indeed, the metal uptake has been shown to be reduced by the presence of ethylenediamine tetraacetate (EDTA), sulphate, chloride, phosphate carbonate and glutamate ions and other compounds which are typically present in tanning wastewaters (Kapoor and Viraraghavan, 1995). In particular, EDTA chelates the metal ions, forming metal-EDTA complexes, with high stability constants; if these are greater than those for metal biosorption sites on the cell wall surface, the biosorption can be expected to be reduced considerably (Kapoor and Viraraghavan, 1995). Moreover, biosorption efficiency depends upon many factors, including the capacity, affinity and specificity of the biosorbents and their physical and chemical conditions in effluents (Ahluwalia and Goyal, 2007).

As a consequence, the biosorption process is easy to understand when it refers to a single metal aqueous solution. In a multi-ion situation the assessment of sorption becomes complicated and the biosorptive equilibrium, as described by single-solute models, may not represent the true equilibrium of metals for the actual tanning effluents (Kapoor and Viraraghavan, 1995). Our results have pointed out the difficulty of relating the data obtained by the biosorption experiments with metal aqueous solutions and those obtained with real tanning wastewaters. It must also be born in mind the peculiarities of the tanning wastewater used, which with respect to a high salts content, had a very low chromium concentration, having been previously subjected to the traditional physical, chemical and biological treatments effective in removing most of the chromium initially present.

In attempt to make a comparison, the same biomasses were tested for Cr(III) removal from synthetic aqueous solutions at different Cr(III) concentrations. As noted previously, again the chemical pre-treatment of the biomass improved the biosorption yields at all the synthetic solution concentrations; besides, the number of ions adsorbed from higher concentrations was more than that removed from less concentrated solutions. This last result is in accordance with those obtained by Kumar et al. (2008) working with Cr(VI) and can be explained by the fact that a higher ions concentration enhances the mass transfer driving force, and increases the metal ions adsorbed per unit weight of adsorbent at equilibrium; moreover, increasing metal ions concentration increases the number of collisions between metal ions and sorbent, improving the biosorption process (Bai and Abraham, 2002). The monitoring of the pH variation during the Cr(III) biosorption process showed that this parameter decreased proportionally to the amount of Cr(III) removed, suggesting a hypothesis of ion exchange between protons and metal ions as observed also by other authors (Sag, 2001).

The comparison between the two kinds of experiment (with real tanning effluent or synthetic solutions) clearly demonstrates that the difficulties in removing Cr(III) from real tanning effluents are due not only to the low metal content but also, as stated above, to the presence of organic or inorganic complexing matter that sequestrates Cr(III) ions (i.e. EDTA, NTA, tannins, etc.). In fact, biomass pre-treatment (NaOH) and initial Cr(III) concentrations (0.4 mg l^{-1}) being equal, Cr(III) ions in aqueous solution are more available to the fungal biomass than those in real wastewater: both removal percentage and SC value were about two times higher in the case of synthetic solution. Hence, the difference in biosorption yields of the same biomass towards the synthetic and real effluents can be explained by the fact that Cr(III) ions are in two different forms: in the first case they are free and ready available to the biomass, in the second case they are complexed to organic or inorganic matter and probably adsorbed to the biomass in their complexed form. In addition, in real effluents there is the simultaneous presence of different cations competing for the same functional groups of the fungal biomass.

5. Conclusions

In conclusion, the results of this study show that:

C. elegans biomass is endowed with a high ability to adsorb Cr(III) from a real tanning effluent in which the metal content is very low and Cr(III) ions are sequestrated by organic and/or inorganic complexing matter;

the Cr(III) removal yields displayed by the selected biomass are higher than those obtained using conventional chemical physical wastewater treatment (i.e. clariflocculation) or using some purified bio-polymers, that resulted effective for Cr(III) biosorption from aqueous solution, but absolutely ineffective for Cr(III) biosorption from the real tanning wastewaters used (Acque del Chiampo S.p.a., personal communication);

NaOH pre-treatment of the biomass improved the biosorption yields.

Hence, biosorption by means of fungal biomasses could be considered a valid alternative to other techniques for wastewater treatment, being applicable to effluents containing low concentrations of heavy metals. This last aspect makes it even more attractive for treatment of dilute effluent that originates either from an industrial plant or from the primary wastewater treatment facility. However, it must be born in mind that this study is preliminary and indicative in nature. Future investigations are obviously needed to improve the biosorption capability of the biomasses and to understand all the biotic and abiotic factors that affect the biosorption, changing both the cell surface binding sites and the metal chemistry in the effluent. Certainly, a possible application of the method on an industrial scale will be preceded by a phase of the study aimed to optimize the amount of biomass to be used, the incubation time, the system to be adopted (e.g. fixed bed, sequencing batch, continuous feeding strategies, etc.), and, more generally, to the reduction of operating costs.

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