Biosorption of hexavalent chromium by *Termitomyces clypeatus* biomass: Kinetics and transmission electron microscopic study

Sujoy K. Das, Arun K. Guha*

Department of Biological Chemistry, Indian Association for the Cultivation of Science, Jadavpur, Kolkata 700 032, India

ARTICLE INFO

ABSTRACT

Biosorption of Cr^{+6} by *Termitomyces clypeatus* has been investigated involving kinetics, transmission electron microscopy (TEM) and Fourier transform infrared spectroscopic (FTIR) studies. Kinetics experiments reveal that the uptake of chromium by live cell involves initial rapid surface binding followed by relatively slow intracellular accumulation. Of the different chromate analogues tested, only sulfate ion reduces the uptake of chromium to the extent of ~30% indicating chromate ions accumulation into the cytoplasm using sulfate transport system. Metabolic inhibitors, e.g. N,N'-dicyclohexylcarbodiimide, 2,4-ditrophenol and sodium azide inhibit chromate accumulation by ~30% in live cell. This indicates that accumulation of chromium into the cytoplasm occurs through the active transport system. TEM-EDXA analysis reveals that the chromium localizes in the cell wall and also in the cytoplasm. Reduction of chromate ions takes place by chromate reductase activity of cell-free extracts of *T. clypeatus*. FTIR study indicates that chromate ions accumulate into the cytoplasm and then reduced to less toxic Cr^{+3} compounds.

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1. Introduction

Chromium, a toxic heavy metal, dissipates into the environment as a result of various industrial activities [1,2]. In view of toxicity and related environmental hazards [3], it is essential that the concentration of chromium in the effluent must be brought down to permissible limit [4] before discharging into water bodies. Among different available technologies [5,6] the removal of metal ions from wastewater by adsorption on biological materials specially microbial biomass known as biosorption/bioaccumulation [7–10] has recently gained much importance. This method does not generate toxic sludge, capable of reducing the concentration of metal ions below the permissible limit and the possibility of regeneration of the materials and thus provide an effective and economic means for the remediation of heavy metal polluted wastewater [11–14]. The uptake of heavy metals by microbial biomass is essentially a biphasic process consisting of metabolism independent initial cell surface binding that can occur either in living or inactivated organisms, followed by energy dependent intracellular accumulation which takes place only in the living cells [15]. The cell wall materials are involved in the initial surface binding of metal ions though electrostatic, physical and/or chemical interaction [16,17]. In living cells besides surface adsorption, metal ions may enter into the cytoplasm through specific carrier system. The transport process in prokaryotic organisms has been studied in some details [18–22]. The state of art in the field of biosorption of heavy metals has recently been reviewed by Volesky [23]. However, only a few reports are available on fungal systems [24,25]. Fungal biomass has certain advantage over bacterial biomass in this natural 'ecofriendly green technological process' in respect of processing and handling of the biomass. Further, in comparison to bacteria, fungi are known to secret much higher amount of exopolymers, thereby significantly increasing the productivity of biosorption/bioremediation process [26]. In this manuscript we describe the biosorption/or bioaccumulation mechanism of chromium on *Termitomyces clypeatus* biomass (TCB) from kinetics study in presence of different co-ions and metabolic inhibitors with support from Fourier transform infrared spectroscopy and transmission electron microscopic investigations.

2. Materials and methods

2.1. Chemicals

Dehydrated microbiological media and ingredients were procured from Himedia, India. All other reagents were of analytical grade and obtained from Merck, Germany and Sigma, USA.

2.2. Metal solution and analysis

A stock solution of chromium (100 mg/l) was prepared by dissolving potassium dichromate ($K_2Cr_2O_7$) in double distilled water

* Corresponding author. Tel.: +91 33 2473 4971X502; fax: +91 33 2473 2805.
E-mail addresses: bcakg@iacs.res.in, arunkumarguha@yahoo.com, bcakg@mahendra.iacs (A.K. Guha).

and diluted to get the desired concentration. The concentration of chromium was measured by atomic absorption spectrometer (Varian Spectra AA 55).

2.3. Biosorbent preparation

Termitomyces clypeatus used in this study was kindly supplied by Dr. S. Sengupta, Indian Institute of Chemical Biology, Kolkata, India, and was grown in complex medium described earlier [27]. Biomass was harvested from the fermented medium by centrifugation (Sorval RC-5B refrigerated centrifuge) at 10,000 rpm for 10 min at 4 °C and washed with deionized water. Biomass was then dried by lyophilization. Dead biomass was prepared by autoclaving the biomass at 121 °C.

2.4. Batch experiment

Biosorption experiments were conducted with 0.2 g lyophilized live and dead *T. clypeatus* biomass (TCB) and 25 ml of $K_2Cr_2O_7$ solution containing 100 mg/l chromium taken in 100-ml Erlenmeyer flask, and incubated at 30 °C (ambient temperature) for 48 h with constant shaking (130 rpm) unless otherwise stated. The solution pH was 3.0 (ionic strength ~0.001 M), being optimum for chromium adsorption. Chromium (VI) may be present in aqueous solution in different oxyionic entities depending on the solution pH [28]. Hydrogen chromate and dichromate occur together at pH value 3.0, whereas dihydrogen chromate is a significant species at pH value 1.0. At the end of incubation, biomass was separated by centrifugation (10,000 rpm for 10 min) and chromium concentration in the supernatant was measured. The uptake of chromium by the biomass was calculated using the mass balance equation [29] and also after digestion of the chromium loaded biomass with aqua regia (HCl:HNO$_3$; 3:1).

The influence of other anionic species on the uptake of hexavalent chromium in presence of 100 and 500 mg/l sulfate (Na_2SO_4), nitrate ($NaNO_3$), phosphate (Na_2HPO_4), arsenate (Na_3AsO_4) and molybdate ($Na_2MoO_4 \cdot 2H_2O$) by TCB was carried out at pH 3.0. Corresponding mM concentrations of the anions were: sulfate, 1.042 and 5.208; nitrate, 1.613 and 8.065; phosphate, 1.054 and 5.269; arsenate, 0.719 and 3.599; molybdate, 0.265 and 3.127. The concentration of chromium was 100 mg/l. To study the effect of metabolic inhibitors or ionophores on chromium adsorption, live TCB was

incubated initially in 50 mM acetate buffer (pH 7.0) at 30 °C for 30 min individually with 200 μM N,N′-dicyclohexylcarbodiimide (DCCD), 1 mM sodium azide (NaN_3) and 1 mM 2,4-dinitrophenol (DNP). The biomass incubated in 50 mM acetate buffer (pH 7.0) served as the control. After incubation biomass was collected by centrifugation, washed with deionized water and used for adsorption experiments at pH 3.0 as described above.

The kinetics of chromium uptake by metabolic inhibitor treated or untreated TCB was followed at regular time intervals up to 48 h using 100 mg/l chromium concentration at pH 3.0. The samples were collected from individual flask; as such, no correction was necessary due to withdrawal of the sampling volume.

2.5. Transmission electron microscopy and energy dispersive X-ray analysis (TEM-EDXA)

The samples of TCB before and after chromium uptake for transmission electron microscopy were prepared as described earlier [29]. Micrographs were recorded on HRTEM (JEOL JEM 2010) instrument equipped with energy dispersive X-ray analysis (EDXA). TEM data were analyzed from multiple samples.

2.6. Detection of chromium in the cytoplasm

T. clypeatus cells after adsorption of chromium were harvested by centrifugation at 5000 rpm for 10 min at 4 °C. The pellets were thoroughly washed with deionized and double distilled water, and then disrupted mechanically with sea sand at 4 °C. The disrupted cells were suspended in phosphate buffer (pH 7.2) and centrifuged at 10,000 rpm for 20 min at 4 °C. After centrifugation, supernatant were collected and drop casted in the form of film on Si (1 1 1) substrates and then dried. The dried films were then characterized by Fourier transform infrared spectroscopy (Nicolet-Magma 750 FTIR spectrometer) in the region of 400–2000 cm^{-1}. The FTIR spectra were recorded with 500 scans at a resolution of 2 cm^{-1}.

2.7. Chromate reductase activity

T. clypeatus biomass obtained after harvesting from the growth medium was thoroughly washed with deionized and double distilled water and disrupted with sea sand in a mortar and pestle at 4 °C. This was suspended in 50 mM phosphate buffer (pH 7.0) and centrifuged at 10,000 rpm for 20 min at 4 °C. Chromate reductase activity in the supernatant containing ~1 mg protein/ml was measured following the method of Ishibashi et al. [30] using 10 mg/l of Cr^{+6} solution, 200 μM NADH and 1 h incubation time at 30 °C. Concentration of Cr^{+6} in the reaction mixture was determined by diphenylcarbazide [30].

3. Results and discussion

3.1. Chromium uptake and effect of metabolic inhibitor

The chromium uptake capacity by both live and dead TCB was studied initially in batch process to understand the biosorption mechanism. Initial batch biosorption experiment with 100 mg/l of chromium shows that 1 g live TCB accumulate 11.1 mg of chromium, while dead biomass accumulate 6.75 mg under the same experimental conditions (Table 1). The reduced uptake of chromium by dead biomass may be due to either loss of some binding sites resulting from heat inactivation of cells or restraint of intracellular chromium accumulation as in the case of viable cells.

Energy dependent transport of many divalent cations has been demonstrated in different microorganisms [31–37]. Divalent metal cation uptake may be energized by the H$^+$ gradient, as found for

Table 1
Effect of different co-ions and metabolic inhibitors on accumulation of chromium by *T. clypeatus* biomass.

Treatment	Uptake (mg/g)[b]	% inhibition
Live TCB[a]	11.1 ± 0.21	–
Dead TCB[a]	6.75 ± 0.25	39.19 ± 2.5
Live TCB + SO$_4^{-2}$ (100 mg/l)[c]	7.59 ± 0.22	31.62 ± 2.0
Live TCB + SO$_4^{-2}$ (500 mg/l)[c]	7.21 ± 0.17	35.05 ± 1.5
Live TCB + AsO$_4^{-3}$ (100 mg/l)[c]	10.17 ± 0.15	8.38 ± 1.5
Live TCB + AsO$_4^{-3}$ (500 mg/l)[c]	10.07	9.2 ± 1.8
Live TCB + MoO$_4^{-2}$ (100 mg/l)[c]	10.41 ± 0.11	6.22 ± 1.1
Live TCB + MoO$_4^{-2}$ (500 mg/l)[c]	10.15 ± 0.18	8.55 ± 1.8
Live TCB + PO$_4^{-3}$ (100 mg/l)[c]	10.26 ± 0.17	7.57 ± 1.7
Live TCB + PO$_4^{-3}$ (500 mg/l)[c]	9.81 ± 0.2	11.62 ± 2.0
Live TCB + NO$_3^{-1}$ (100 mg/l)[c]	10.72 ± 0.15	3.42 ± 1.5
Live TCB + NO$_3^{-1}$ (500 mg/l)[c]	10.31 ± 0.2	7.12 ± 2.0
Live TCB + 200 μM DCCD[d]	7.19 ± 0.19	35.2 ± 2.0
Live TCB + 1 mM DNP[d]	7.61 ± 0.27	31.44 ± 2.5
Live TCB + 1 mM NaN$_3$[d]	8.34 ± 0.33	24.89 ± 3.0

[a] No competitive ion or inhibitor was added.

[b] Data represent an average of five independent experiments ± SD shown by error bar.

[c] Accumulation of chromium by live *T. clypeatus* biomass was carried out in the presence of competitive ion.

[d] Live *T. clypeatus* biomass was pre-incubated with metabolic inhibitors.

Cd^{+2} and Ni^{+2} uptake in yeast [38]. A detailed study on transportation of chromium have been reported in prokaryotic organisms, however, the mechanism for chromium transport is not adequate in fungal biomass. Live TCB was incubated in presence of different metabolic inhibitors to gain a better understanding of the energy requirement in the intracellular accumulation of chromium. All the inhibitors, e.g. DNP (uncoupler), DCCD (ATP synthetase inhibitor) and NaN_3 (terminal oxidase inhibitor) significantly reduced Cr^{+6} uptake (Table 1). Uncouplers of oxidative phophorylation prevent ATP synthesis [39] in mitochondria by dissipating the energized membrane state while substrate oxidation and oxygen consumption proceed normally. Thus it is expected that the active transport process which requires energy would be inhibited where primary source of ATP generation is oxidative phophorylation. Inhibition of chromium uptake by DNP (uncoupler) to the extent of ∼30% indicates that ATP generated by oxidative phosphorylation is required in this process. DCCD, an inhibitor of proton translocating plasma membrane P-type ATPase, inactivates the ATP synthetase function [34,39] by inhibiting proton translocation through the F_0 subunit of the enzyme. This compound also inhibited (∼35%) chromium

uptake almost to the same extent as DNP, indicating involvement of the H^+/ATPase [32] for H^+ efflux during chromium uptake. Hence, a P-type ATPase, probably located on the plasma membrane, might be directly involved in chromium transport. This ATPase is important for generating a proton gradient across the plasma membrane, which drives transport of chromate into the cytoplasm of the cell. The respiratory chain inhibitor, NaN_3 [39], also lowered the chromium uptake to the extent of ∼25%. These results demonstrate that transportation of chromium is an energy-dependent process that is driven by a proton motive force. The dead biomass, which contains no ATP, adsorbed/accumulate lesser amount (∼40%) of chromium supporting the above view of phosphate-bond energy involvement in the intracellular accumulation of chromium.

3.2. Effect of chromate analogue on accumulation of chromium

Transportation of chromate by structurally similar sulfate active system has been reported in bacterial system [40,41] but remain unexplored in the fungal cells. In general, toxic ions having close chemical similarities to nutrient ions are mistakenly accumulated
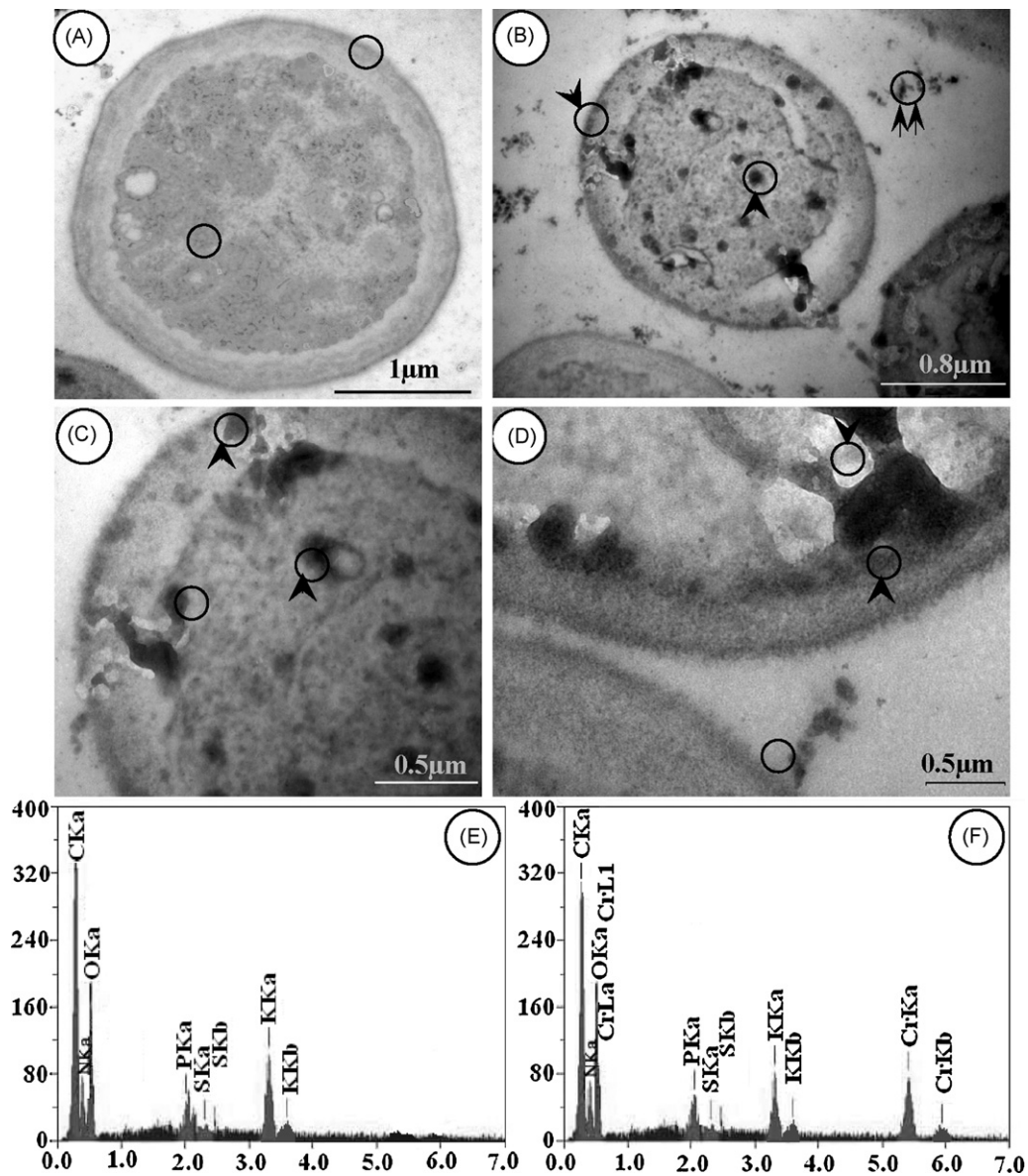

Fig. 1. Transmission electron micrographs of pristine biomass (A); chromium adsorbed live biomass: (B) low magnification, (C and D) high magnification. EDXA spectra of pristine (E) and chromium adsorbed biomass (F). EDXA spectra were recorded from the marked area.
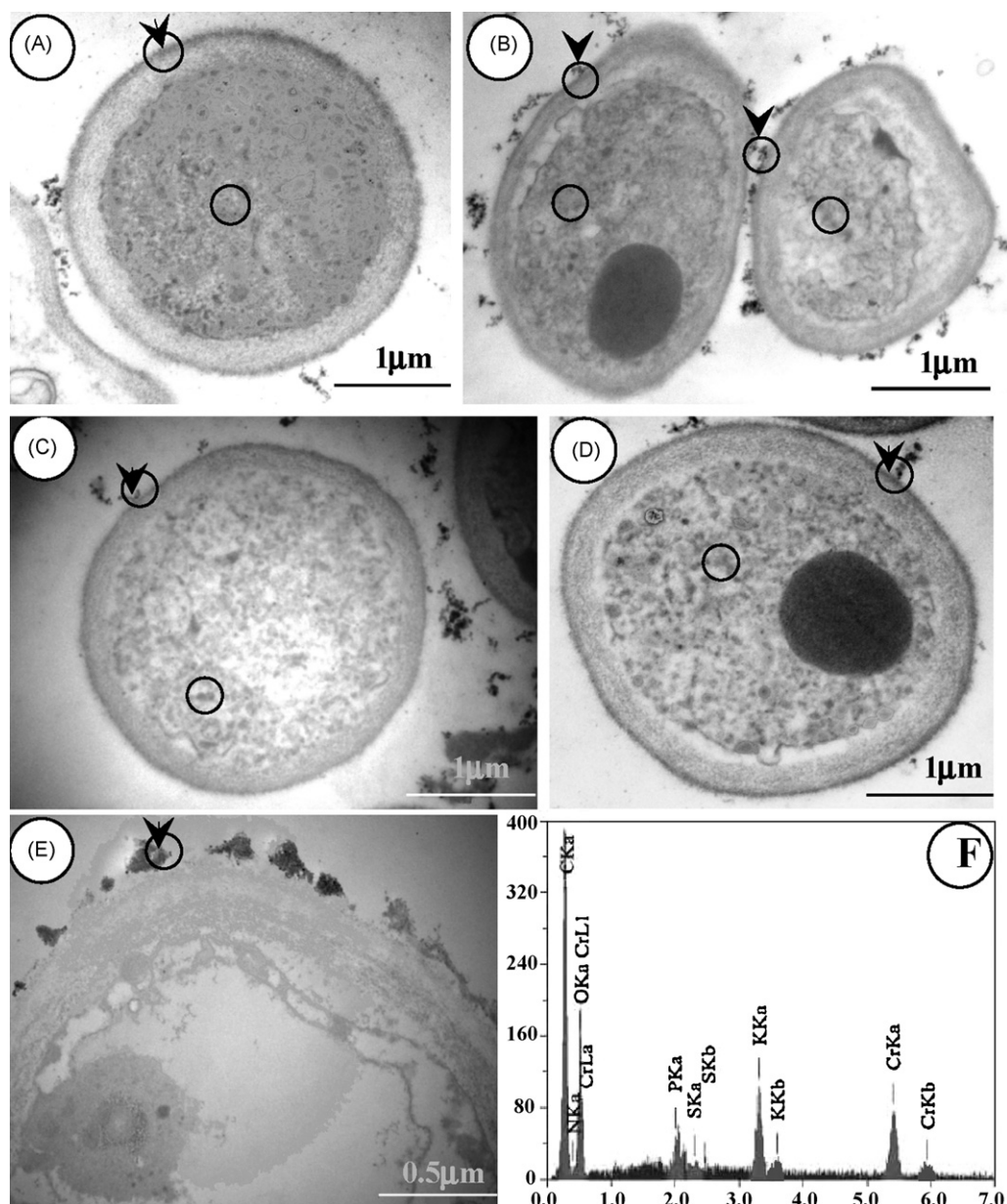
Fig. 2. Transmission electron micrographs of chromium adsorbed biomass in presence of sulfate ions (A); chromium adsorbed on metabolic inhibitor treated biomass (B–E); (B) sodium azide, (C) DNP, (D) DCCD treated biomass, and (E) high magnification of inhibitor treated post adsorbed biomass. (F) EDXA spectra of chromium adsorbed with metabolic inhibitor treated biomass.

by cells as it happens in the case of cadmium–manganese [42] and arsenate–phosphate [43] systems. The uptake of chromium by live biomass was reduced by 30–35% (Table 1) in presence of sulfate ion but remain unchanged in presence of other anionic species such as phosphate, nitrate, molybdate, and arsenate. Thus a direct competition between chromate and sulfate ions caused reduction in the uptake of chromium by live biomass which suggests that chromate was accumulated within the cell by the sulfate transport system as it occurs in *P. fluorescence* [40]. These observations suggest that chromium was accumulated into the cytoplasm of *T. clypeatus* cell by active sulfate transport system.

3.3. Transmission electron microscopic study and energy dispersive X-ray analysis (TEM-EDXA)

To understand the mechanism of complex metal–microbes interactions, it is important to determine the location of the

chromium relative to the fungal cells. Transmission electron micrograph of the thin section of Cr^{+6} adsorbed biomass (Fig. 1B) exhibits electron dense granules on the cell wall as well as within the cytoplasm; whereas in control cells these are absent (Fig. 1A). Micrographs at higher magnification (Fig. 1C and D) of the post adsorbed biomass show the presence of chromium on the cell wall (outer boundary), periplasmic space, cytoplasmic membrane (inner boundary), and also within the cytoplasm of live TCB. Elemental analysis as provided by EDXA showed that the electron dense granules are composed of chromium. The spectrum (Fig. 1F) shows the presence of chromium peak in both cell wall and cytosolic region of the live cell. Chromium rich granules are also found to associate with the extracellular polymers secreted by live cells (Fig. 1B) as indicated by double arrow in the micrographs. No chromium peak (Fig. 1E) is detected in the pristine cells.

Intracellular transportation of chromium is restricted in the different metabolic inhibitor treated TCB; indeed, chromium is
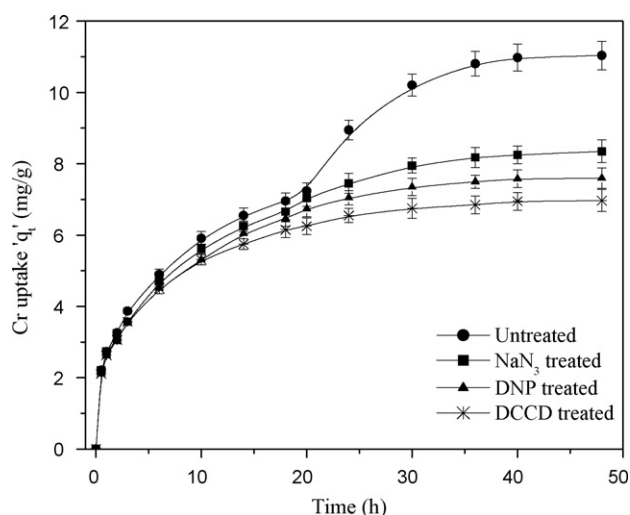
Fig. 3. Chromium uptake at different contact times by (-●-) untreated, (-■-) azide treated, (-▲-) DNP treated, and (-✳-) DCCD treated *T. clypeatus* biomass; Data represent an average of five independent experiments ± SD shown by error bar.

confined only to the cell wall (Fig. 2B–D). Magnified micrograph (Fig. 2E) further supports the above observation. Chromium peak is detected only in the cell wall portion (Fig. 2F) but not in cytosolic region of metabolic inhibitor treated TCB (figure not shown). It is also delineated from TEM-EDXA analysis that transport of chromate into intracellular organelles is restricted in presence of sulfate ions (Fig. 2A). The arrows in the micrographs indicate the location of the metal ion. The appearance and distribution of chromium in both cell wall and cytoplasm of the untreated biomass indicate that chromate ions initially adsorbed on the cell wall and then accumulated into the cytoplasm. Since electron dense granules are formed on the cell wall as well as with the cytoplasm of the live *T. clypeatus* cells, we propose the first step involves the binding of chromate ion on the surface of the cells. This could occur by electrostatic interaction with the positively charged functional groups of the cell wall, thereafter transported into the cytoplasm through different layers (chitin-chitosan, glucan, and mannan) [44] of the cell wall and cytoplasmic membrane by energy dependent transport system for sulfate ions. It is possible that enzymes present in the cytoplasm reduce chromate to Cr(III) compound.

3.4. Kinetic study of chromium accumulation

The rate of uptake as well mechanism can be monitored from kinetic studies. In general, microorganisms accumulate metal ions by a two phase process: (i) initial rapid, reversible or irreversible metabolism independent binding on the cell surface followed by (ii) relatively slow energy dependent intracellular accumulation by specific carrier systems [45]. Another mechanism may also be operative in active cells, i.e., extracellular precipitation of insoluble metal species as a result of metabolically produced carbonates, hydroxides and sulfides. To confirm the requirement of energy in the intracellular accumulation of chromium in TCB, the biomass was incubated initially with NaN$_3$ (terminal oxidase inhibitor), DNP (uncoupler) or DCCD (ATP synthetase inhibitor) and were used to study the kinetics of hexavalent chromium uptake process. Kinetic study showed that live (untreated) TCB accumulated more chromium compared to the metabolic inhibitor treated TCB (Fig. 3). Chromium uptake was initially very fast and then gradually slowed down to reach equilibrium after 40 h (Fig. 3). The rate of chromium uptake by TCB was slow compared to previous reports on metal ion uptake by bacteria [46,47], probably due to large surface area of bacterial cells.

The rate or extent of chromium accumulation was significantly altered in presence of different metabolic inhibitors. The bi-phasic nature of the chromium uptake curve in live TCB indicates initial cell surface binding followed by intracellular accumulation as reported earlier by Baldi et al. [45] in *S. cerevisiae*. The initial rapid rate was probably due to the availability of abundant binding sites on the cell surface, which became saturated after a certain period of time resulting in the decreased rate of uptake. Following this binding phase, comparatively slow intracellular accumulation of chromium
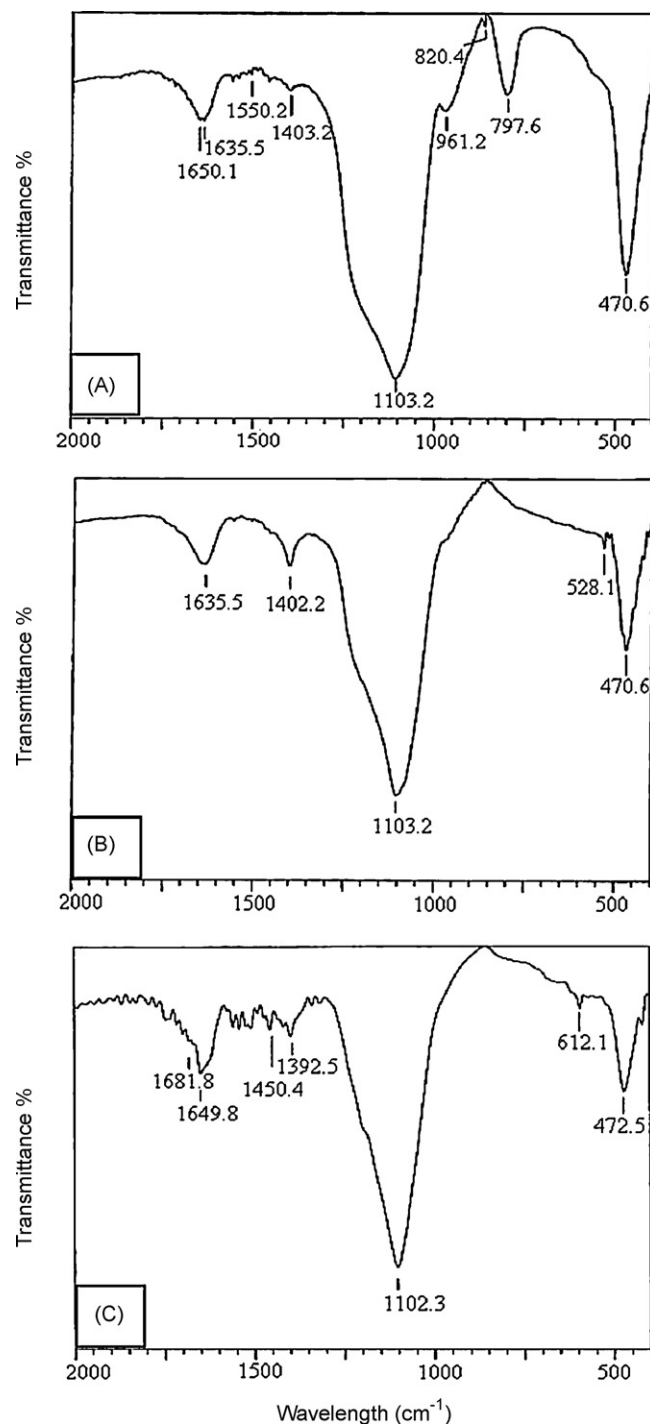

Fig. 4. The FTIR spectra of the cell-free extract obtained (A) after adsorption of chromium for 24 h, (B) after increasing adsorption time to 48 h and (C) IR spectrum of the cell-free extract obtained for adsorption experiments carried out in presence of sulfate ions.

occurred either through passive diffusion [48] or energy dependent accumulation by specific carrier system [34,35]. The results (Fig. 3) indicate that the uptake of chromium by both metabolic inhibitors treated or untreated biomass are almost equal up to certain time. However, main difference in the kinetics curve observed at $t > 20$ h. Untreated biomass further accumulates chromium beyond this time period ($t > 20$ h) due to intracellular accumulation but at a slow rate and finally reach equilibrium after 40 h. The absence of second phase in the metabolic inhibitors treated biomass might be due to the inhibition of transportation of chromium into the cytoplasm. This indicates that after initial surface binding phase, metabolism dependent transport of chromium occurred in *T. clypeatus* biomass. The initial surface binding phase occurs through metabolism independent pathway as there is no difference observed in uptake curve at $t < 20$ h.

3.5. Fourier transform infrared (FTIR) spectroscopic study

Fourier transform infrared (FTIR) spectra were recorded from drop cast film on Si (1 1 1) substrates of the cell-free extract of chromium adsorbed biomass. The cell-free extract shows (Fig. 4) the strong amide I and II absorption bands at 1650 and 1550 cm^{-1} [49,50] respectively, for proteins molecules. The IR spectrum of the cell-free extract obtained after adsorption of chromium for 24 h shows characteristic vibration bands of chromate ions at 961.2, 820.4, and 797.6 cm^{-1} (Fig. 4A) [51] delineate transportation of chromate into the cytoplasm. A new absorption band appears at 528.2 cm^{-1}, characteristic for Cr(OH)$_3$ [52] along with disappearance of band for chromate as well as shifting or disappearance of amide bands after increasing adsorption time to 48 h (Fig. 4B). This demonstrates that after transportation into cytoplasm, chromate ions get reduced to Cr^{+3} compounds by the cytosolic enzymes. However, no characteristic peak of chromium is detected in the cell-free extract obtained after adsorption of chromium with metabolically inhibited biomass (data not shown) or adsorption experiments carried out in presence of sulfate ions (Fig. 4C). It is interesting to note an absorbance band of suphate ions is detected at 612.1 cm^{-1} demonstrate sulfate ions [53] inhibit the transportation of chromate ions. This result indicates that after initial surface binding, chromate ions entered into the cytosol through energy dependent sulfate transport system.

3.6. Chromate reduction by cell-free extracts

It is possible that after transportation of chromate into the cellular organelle, the cytoplasmic enzyme may reduce it to trivalent state. We therefore studied the enzymatic reduction of chromate ions by the cell-free extract of *T. clypeatus*. Cr^{+6} reduction activities were evident in the cell-free system under aerobic conditions. Approximately 80% of a solution of 10 mg/l Cr^{+6} was reduced within

1 h. The cell-free enzyme requires NADH as an electron donor for the reduction of chromate. The reductase activity was lost when the cell-free extract was boiled for 10 min. Since reduction is associated with the cell-free extracts, the cytoplasmic enzyme [54,55] is mainly responsible for reduction of Cr^{+6} to Cr^{+3}. A detailed study on enzymatic reduction of chromium is currently underway.

Our results thus demonstrate that *T. clypeatus* biomass removes chromate ions from aqueous solution initially by adsorption followed by reduction into less toxic Cr^{+3} compound, and thus provide a fungal mediated "green chemical process" for the remediation of chromium from wastewater. A schematic presentation of intracellular accumulation of chromium by *T. clypeatus* biomass is shown in Fig. 5.

4. Conclusions

Kinetics study reveals that the uptake of chromium by live *T. clypeatus* cell follows a two-phase process involving initial rapid surface binding followed by relatively slow intracellular accumulation. The uptake of chromium is reduced in presence of sulfate ion, a chromate analogue and different metabolic inhibitors which delineate that intracellular accumulation of chromium occurs through sulfate active transport system. Transmission electron microscopy equipped with energy dispersive X-ray analysis reveal that the chromium localizes in the cell wall as well as in the cytoplasm. Chromate reductase activity is noted in the cell-free extracts of *T. clypeatus*, is probably responsible for reduction of chromate ions. FTIR study indicates that chromate ions accumulate into the cytoplasm and then reduced to less toxic trivalent chromium compounds. Fungal biomass thus provides a green chemical process for the remediation of chromium from wastewater.

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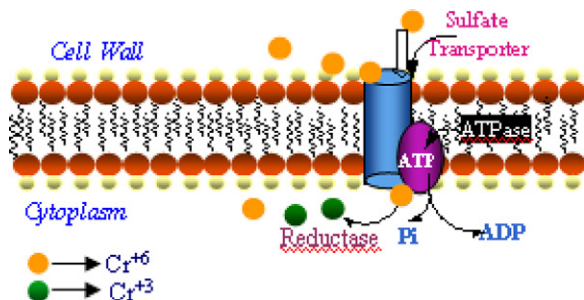

Fig. 5. A schematic presentation of chromium uptake by *T. clypeatus* biomass; chromium initially adsorbed on the cell surface and then transported into cytoplasm through sulfate active transport system.

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