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ORIGINAL ARTICLE

An investigation on tolerance and biosorption potential of *Aspergillus awamori* ZU JQ 965830.1 TO Cd(II)

Manal Tawfeek El-Sayed

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Abstract The minimum inhibitory concentration (MIC) value of *Aspergillus awamori* for Cd(II) was 700 mg/l. A complete inhibition of biomass production was observed at 400 mg/l concentration of Cd(II). A significant deformation in Cd(II)-stressed conidiophores and conidia was observed by Scanning electron microscopy (SEM) investigation. Quantification of Cd(II) was performed by EDX microanalysis. Transmission electron microscopy investigation (TEM) confirmed the involvement of extracellular adsorption, intracellular penetration through the cell wall and vacuolation. Cadmium(II) stress induced noticeable changes in the activities of polyphenol oxidase (PPO), glutathione reductase (GR) and peroxidase (POD), and in the concentrations of total antioxidants, soluble protein and thiols. High performance liquid chromatography analysis (HPLC) revealed that Cd(II) stress stimulated the production of oxalic acid. Maximum Cd(II) uptake capacity was achieved at pH 5.0, initial metal ion concentration 500 mg/l and biomass dosage 1 g/l. Maximum Cd(II) uptake capacity was reached after 6 h for live biomass and after 2 h for dead biomass. Fourier transform infrared spectroscopy (FTIR) results gave an indication on chelation between oxygen-, nitrogen-, phosphorus- and especially sulphur-containing ligands of biomass with metal ions. X-ray diffraction analysis (XRD) revealed the presence of $CdSO_4 \cdot H_2O$ by live and dead biomass. EDX confirmed the occurrence of sulphur, oxygen and Cd(II) on the cell wall.

Keywords *Aspergillus awamori* · Cd(II) · Stress · Antioxidant enzymes · Biosorption

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M. T. El-Sayed (✉)
Botany Department, Faculty of Science, Zagazig University, Zagazig, Egypt
e-mail: tawfeek.manal1@gmail.com

Introduction

Environmental pollution as a result of rapid technological development is a serious concern for ecology. Heavy metal ion contamination represents a significant threat to the ecosystem (Hajiaghababaeli et al. 2011). The main sources of heavy metals pollution include electroplating, painting, dying, and surface treatment industry. Biodegradation of heavy metals is not possible because, unlike organic pollutants, they do not biodegrade to non-toxic compounds such as H_2O and CO_2 (Ramanthan and Muthukkaruppan 2007). Some metal ions, like Cu(II), are essential for life, but other metals like Cd(II) are considered highly toxic elements to nearly all organisms even at low concentrations (Cardoso et al. 2002). According to World Health Organization (WHO), the metals of immediate concern are Cd(II), Hg(II) and Pb(II). Cadmium(II) damages cells by strong affinity to glutathione and sulfhydryl groups in proteins, displacement of zinc and iron ions from proteins, interaction with calcium metabolism and membrane damage and generation of reactive oxygen species (ROS) (Banjerdkij et al. 2005). Research points to a relationship between Cd(II) and reactive oxygen species and cancer (Ziad et al. 2008). Cadmium(II) can also inhibit the growth of many fungi (Soares et al. 2003).

Although the removal of toxic heavy metals from industrial waste waters has been practiced for several decades, the most common physicochemical processes such as oxidation and reduction, chemical precipitation, filtration, electrochemical treatment, evaporation, ion-exchange and reverse osmosis processes are not very cost-effective (Malik 2004). Sorption of heavy metals onto biosorbents is the uptake from aqueous solutions or from effluents by biological materials. Biosorption is proven to be an effective method because it covers a large surface area, uses an effective ion exchange, has selective adsorption of heavy metal ions, is cheap but valuable, can be operated over a broad range of environmental

conditions, is a fast and reversible reaction that is ecofriendly in nature and has excellent performance (Issac et al. 2012). Fungi have a high percentage of cell wall material that shows excellent metal-binding properties and exhibit marked tolerance towards metals and other factors such as low pH (Zafar et al. 2007). Recently, heavy metal-accumulating strains have been sought for a new role as small factories for the production of nanoparticles (Klaus-Joerger et al. 2001). The status of biomass (live or dead), types of biomaterial, properties of metal solution chemistry, ambient/environmental conditions such as pH, will all influence the mechanism of metal biosorption. The mechanisms of metal biosorption can be defined according to the location where the metal removed from the solution is found: extracellular accumulation/precipitation, cell surface sorption/precipitation or intracellular accumulation. Microorganisms have evolved various measures to respond to heavy metal stress via transport across the cell membrane, biosorption to cell walls and entrapment in extracellular materials, precipitation, complexation and oxidation-reduction reactions (Veglio and Beolchini 1997).

The purpose of this study was to investigate the response and tolerance mechanism of *A. awamori* to Cd(II) stress. This study was undertaken also to determine the Cd(II) biosorption potential of live (not growing) and dead *A. awamori* biomass under different conditions. The biosorption mechanism was investigated using FTIR, XRD and EDX.

Materials and methods

Microorganism, growth conditions and preparation of biosorbent

Aspergillus awamori ZU JQ 695830.1 was kindly obtained from our laboratory stock cultures (Mycology lab, Botany Department, Faculty of Science, Zagazig University). The isolate was cultured on potato dextrose agar slants (PDA) (Gams et al. 1998) and stored at 4 °C.

For the preparation of the biosorbent, *A. awamori* was cultured on potato dextrose broth (PD). After 5 days of incubation at 30 °C on a rotary shaker at 125 rpm, the fungal growth was harvested, filtered and washed with distilled water to remove residual growth medium. The biomass was divided into two parts; the first one was used as live (not growing) biosorbent while the other part was dried to a constant weight at 60 °C and powdered to be used as a dead biomass in the biosorption experiments.

Metal solutions

Stock metal solutions of Cd(II) were prepared by dissolving appropriate quantities of $CdCl_2$ salt in double distilled water. The stock solutions were diluted further with deionized distilled water to obtain working solutions of different concentrations.

Determination of minimum inhibitory concentration (MIC)

A sterilized solution of $CdCl_2$ was aseptically added to the sterilized PDA medium to get the final concentrations ranged from 0 to 1,000 μg/ml. The plates were centrally inoculated with 5-mm fungal plugs from 4-day-old fungal colonies in three replicates and were incubated at 30 °C for 7 days. MIC was identified as the minimum concentration of Cd(II) that inhibited visible growth of *A. awamori*.

Effect of Cd(II) stress on *A. awamori* growth

To investigate the heavy metal stress response in *A. awamori*, the changes in radial growth, biomass production, the concentration of protein content, total antioxidant and total thiols, and the activity of peroxidase (POD), polyphenol oxidase (PPO) and glutathione reductase (GR) were determined.

To study the effect of Cd(II) on radial growth, the metal ion-treated plates were inoculated as mentioned before for the determination of MIC. The diameters of the fungal colonies were measured in millimeters (mm) at certain intervals.

To study the effect of metal ions on the biomass production of *A. awamori*, the fungus was grown in 250 ml Erlenmeyer flasks containing 50 ml of PD. Sterilized solutions of cadmium(II) chloride were aseptically added to the sterilized medium to get the final concentration ranging from 0–1,000 μg/ml. It was then incubated at 30 °C for 6 days on a rotary shaker at 125 rpm. The biomass was harvested and dried to a constant weight at 60 °C.

The effects of Cd(II) on the concentration of total soluble protein, thiol content and total antioxidant, and on the activity of POD, GR and PPO were determined. The fungal mycelia were ground using a cold mortar in an ice bath with 50 mM cold phosphate buffer (pH 7.0) of 50 mM EDTA. The cell suspensions were centrifuged at 6,000 rpm for 15 min at 4 °C. The supernatants and filtrates were used to elucidate the tolerance mechanism of *A. awamori* to Cd(II).

Polyphenol oxidase (PPO)

Polyphenol oxidase activity was determined according to Bergmeyer et al. (1974). Briefly, the reaction mixture contained 200 μl of enzyme preparation in 0.1 M potassium phosphate buffer (pH 7.0), 0.2 mM guaiacol, 5 U/ml horseradish peroxidase and 10 mM catechol as substrate, in a total volume of 1 ml. The reaction was incubated for 60 min at 30 °C. The reaction was frozen for 10 min, and the developed color was measured at 436 nm. One unit of the enzyme was

expressed as the amount of enzyme that released 1 μmol H_2O_2 per min under optimal assay conditions.

Glutathione reductase (GR)

Glutathione reductase activity was determined spectrophotometrically according to the method of Barata et al. (2000). The reaction mixture contained 3 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM 2-nitrobenzoic acid, 1 mM oxidized glutathione and 0.1 mM NADPH. The reaction was started by the addition of 50 μl of mycelium extract. The changes in absorbance due to the reduction of oxidized glutathione were monitored at 412 nm for 2 min.

Total antioxidant

Concentration of the total antioxidant of the crude fungal extract was determined by the ferric-thiocyanate method (Gupta et al. 2004) with slight modifications. In brief, 1 ml of the crude enzyme preparation was mixed with 0.2 ml of 20 mM ferrous chloride and 0.2 ml ammonium thiocyanate (30 %). After incubation for 10 min, the developed red color was measured at 500 nm.

Protein measurement

The method of Lowry et al. (1951) was used to measure extracellular and intracellular protein.

Assay of total thiol content

For total thiol assay, Ellman's (1959) method was followed. Three milliliters of sample was mixed with 2 ml phosphate buffer (pH 7.0) and 5.0 ml distilled water which was mixed well to get a 10 ml reaction mixture. Twenty microliters of 0.01 M DTNB solution was added to 3 ml of the reaction mixture, shaken well and absorbance was recorded at 412 nm.

Scanning electron microscopy (SEM) analysis

Aspergillus awamori cells were fixed in 2.5 % glutaraldehyde at 4 °C for 24 h and then post-fixed in 1.0 % osmium tetraoxide at room temperature for 1 h (Harley and Ferguson 1990). Samples were then dehydrated in acetone, coated with gold and examined using a Jeol scanning electron microscope (JEM-1200XII) (Regional Center of Mycology and Biotechnology, Cairo, Egypt).

Energy dispersive X-ray (EDX) microanalysis

Metal-loaded live and dead biomass were used for EDX microanalysis using an X-ray microanalyzer (model Oxford 6587 INCA X-sight) connected to a JEOL JSM-5500 LV scanning electron microscope (Regional Center of Mycology and Biotechnology, Cairo, Egypt).

Transmission electron microscopy (TEM) analysis

Aspergillus awamori cells were fixed in 2.5 % glutaraldehyde for 3 h (Gupta and Berridge 1966), washed twice with 0.2 M phosphate buffer of pH 7.4 for 30 min, then post-fixed in 1.0 % osmium tetraoxide for 2 h (Palade 1952). After that, the cells were washed with phosphate buffer for 30 min. All the previous steps of fixation were carried out at 4 °C. Samples were dehydrated in a graded ethanol series (50 %–100 %). They were passed through three changes of acetone:ethanol (1:2, 1:1 and 2:0) for 10 min each and embedded in epoxy medium (Epon 812) (Luft 1961). Blocks were sectioned with a diamond knife (RMC Products, Boeckeler Instruments, Tucson, AZ, USA) into ultrathin sections of about 70 nm. These ultrathin sections were constructed with uranyl acetate (Sptempack and Ward 1969) followed by lead citrate (Reynolds 1963), each for 30 min. Transmission and photographing were done using a JEOL-1010 electron microscope (Regional Center of Mycology and Biotechnology, Cairo, Egypt).

High performance liquid chromatography (HPLC)

To investigate the role of oxalic acid in the tolerance mechanism, analysis of oxalic acid in control and Cd(II)-stressed samples was carried out using an HPLC system that comprised a GBC UV/vis detector, GBC LC 1110 pump controlled by WinChrome chromatography ver 1.3 software. The eluent was 85 % acetonitrile to 15 % water, and the column used was a Kromasil 100* 4.6 mm with a flow rate of 1 ml/min. The detection was at 254 nm (Regional Center of Mycology and Biotechnology, Cairo, Egypt).

Batch biosorption studies

To investigate the relationship between tolerance and biosorption potential of *A. awamori* to Cd(II), batch biosorption experiments were carried out. In addition, a comparison between live (not growing) and dead biomass was also studied.

All uptake experiments were performed by suspending the biosorbent in 100 ml of solution at desirable pH, biosorbent dose, initial metal ion concentration and contact time. Sorption contact experiments with metal bearing solutions were run in triplicate.

Effect of initial pH

To evaluate the effect of pH on metal uptake, the pH of the solution was adjusted in the range of 2 to 6, viz. 2, 3, 4, 5 and 6, before mixing biomass. The pH was adjusted to the required value with an aqueous solution of 0.1 N HCl or 0.1 N NaOH. Initial metal ion concentration was 500 mg/l, biosorbent dose was 1 g/l at 28 °C and contact time was 120 min (dead biomass) and 360 min (live biomass). The concentration of unadsorbed Cd(II) in the supernatant was measured using an atomic adsorption spectrophotometer (Model Unicam 969, Centric Laboratory, Faculty of Agriculture, Zagazig University).

Effect of initial metal ion concentration

The batches were set at different initial metal ion concentration of Cd(II). Aliquots (50 ml) of 150, 200, 300, 500 and 700 mg/l concentrations of Cd(II) were added to 1 g/l biomass at 28 °C in 500 ml Erlenmeyer flasks.

Effect of biosorbent dose

Live and dead biomass of *A. awamori* with concentrations of 1, 2, 3 and 5 g/l (with respect to cell dry weight) were added to 50 ml of a 500 mg/l concentration of Cd(II), and shaken on a rotary shaker incubator at 28 °C and 125 rpm.

Effect of contact time

The biomass concentration (1 g/l) was exposed to 500 mg/l Cd(II) for different periods of time. Samples were analyzed at intervals of 0, 0.17, 0.50, 1.00, 1.30, 2.00, 3.00, 4.00, 5.00, 8.00, 16.00 and 24 h and the adsorption profile was monitored. For all graphical representations, the mean values of three replicates of the batch experiments were plotted.

Biosorption data evaluation

The amount of metallic ions biosorbed per gram of biomass (q) was determined using the following equation:

$$\text{Biosorption capacity } (q) = \frac{C_i - C_f}{M}\ V$$

where C_i is the initial metal ion concentration (mg/l), C_f is the final metal ion concentration (mg/l), M is the mass of the biosorbent (g), V is the volume of the metal solution and q is the biosorption capacity (mg/g).

Fourier transform infrared spectroscopy (FTIR)

Infrared spectra of native cells as well as live and dead treated samples were recorded over the region 400–4,000 cm^{-1} with a PerkinElmer FTIR 1650 spectrophotometer. The samples were examined in KBr containing 3 % (W/W) of finely ground powder of each sample (Center of Microanalysis, Cairo University, Cairo, Egypt).

X-ray powder diffraction (XRD) analysis

X- ray diffraction pattern of powder samples of metal-free and metal-loaded live and dead biomass were recorded in a Broker D8 Advanced target Cu Koα powder diffractometer (λ=1.5418 A°) over the range of 0–60 2θ (Central Metallurgical & Development Institute, Helwan, Egypt).

Results and discussion

Heavy metal tolerance

The sensitivity of *A. awamori* to Cd(II) was evaluated by MIC. *Aspergillus awamori* showed a limited tolerance to Cd(II) concentration up to 700 mg/l which is about 140,000-fold over the maximum acceptable limit permitted in drinking water (0.005 mg/l). MIC was found to be species-specific and metal-dependent (Ahmad et al. 2006). The MIC values of *A. awamori* in this study are very much higher than those reported by Akhtar et al. (2013). They reported that MIC of Cd(II) for *A. niger* GF-1 and GF-5 was 56 mg/l. Tolerance of heavy metals is based on ionic species associated with the cell surface or extracellular polysaccharides, proteins and chitins (Iram et al. 2009). Among the mechanisms for heavy metal resistance is the segregation of cations, especially the sulfur lovers (e.g. cadmium), into complex compounds by thiol-containing molecules (Nies 1999).

Effect of Cd(II) on the growth of *A. awamori*

Heavy metal toxicity influences several aspects of the fungal growth as the lag phase of the fungi, their growth rate, the density of the mycelium and the biomass production (Jones and Hutchinson 1988). The influence of Cd(II) on the fungal growth that was assessed in terms of colony diameter and mycelia dry weight over a 6-day incubation (Fig. 1Sa and b). The inhibitory effect of Cd(II) was very pronounced; *A. awamori* growth was partially inhibited even by the lowest Cd(II) concentration of 50 mg/l, especially in liquid media, where no black conidia were formed. The growth inhibition was markedly increased with increasing Cd(II) concentration up to 600 mg/l on solid media and 300 mg/l on liquid media. A complete inhibition in colony extension and biomass production was observed at 700 and 400 mg/l concentrations of

Cd(II), respectively. The effect of Cd(II) on the growth curve of *A. awamori* consisted of two components: the extension of the initial lag phase and the reduction of radial growth and biomass production. The long lag phase could be the cause of the inability of the fungus to grow at high concentrations of Cd(II), since the adaptation to growth in a metal-containing medium has to occur prior to the death of the mycelium (Baldrian and Gabriel 2002). The inhibitory effect of Cd(II) may be due to its strong affinity to glutathione and sulfhydryl groups in proteins and displacement of zinc and iron ions from proteins (Banjerdkij et al. 2005). Growth reduction is a typical response of fungi to the toxicity of heavy metals (Baldrian 2003). In a liquid medium, the metal toxicity becomes more evident when the medium is supplemented with lower concentrations than agar plates. This apparent abnormality is due to the more intimate contact between the cells and the ions and also due to the absence of the protective chelating effect of the agar (Ruta et al. 2010).

Assessment of *A. awamori* morphological changes in response to Cd(II) stress and the quantification of these metal ions within the fungal strain were performed by SEM investigation and EDX microanalysis (Figs. 1 and 2, respectively). Control biomass had ordinary, heavily conidiated vesicles with smooth conidiophore (Fig. 1a) and normal conidia (Fig. 1b). In the case of Cd(II) stress, conidiophores were progressively deformed and curled (Fig. 1c and d). There was a complete inhibition in both sterigmata and conidia formation (Fig. 1e). In some Cd(II) treated biomass, there were granules on the conidiophores (Fig. 1f). Toxic heavy metals can inhibit growth, cause morphological changes and affect the reproduction of organisms. The reproductive stage of spore formation and conidia production are much more sensitive to heavy metals than mycelia growth in saprophytic and mycorrhizal soil fungi (Ali 2007).

Biosorption of Cd(II) was confirmed by EDX microanalysis which revealed the presence of Cd(II) signals along with

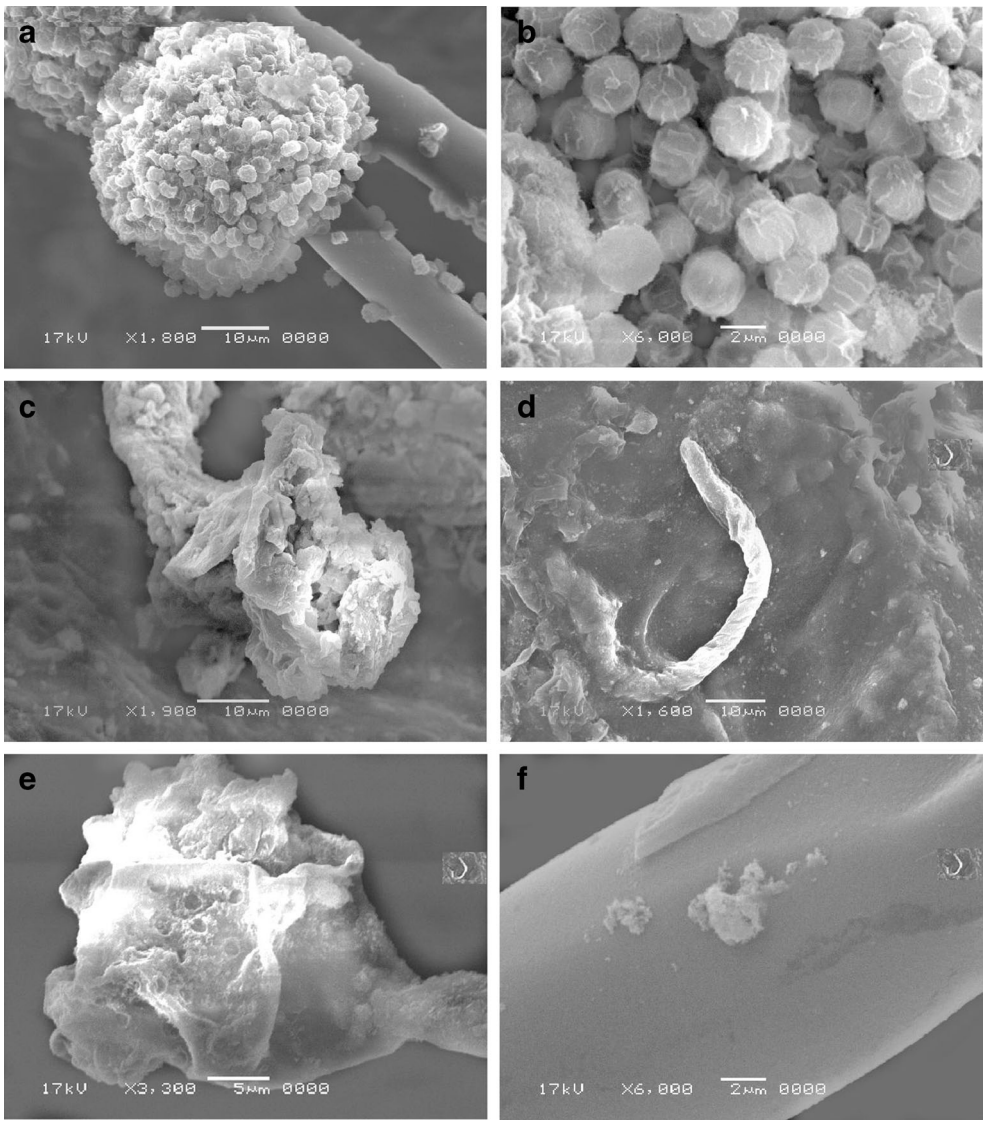

Fig. 1 SEM of *A. awamori* (**a** and **b**) native cells, (**c**–**f**) Cd(II)-stressed cells

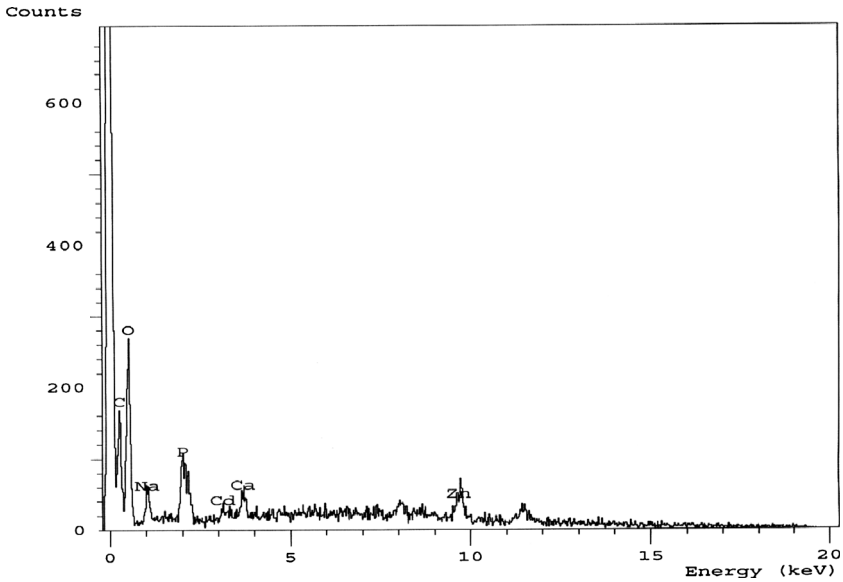

Fig. 2 EDX of *A. awamori* Cd(II)-loaded cells

carbon (C) and oxygen (O) on the metal-loaded fungal biomass (Fig. 2). Comparable results were obtained by Das and Guha (2007).

Aspergillus awamori biomass before and after Cd(II) exposure was subjected to TEM investigation to ascertain the cellular localization of accumulated metals (Fig. 3a–f). Ultrathin sections of metal-less control cells revealed a distinct cell wall 400 nm in thickness, clear cytoplasm with normal mitochondria and few electron-dense areas probably representing the genetic material and cytoplasmic deposits (Fig. 3a). Septum thickness was found to be 220 nm (Fig. 3b). Cadmium(II)-loaded cells exhibited dark, thick, electron-opaque regions bound tightly to the outer layers of the cell wall, within the cell wall layers, and the cell interior indicating homogenous extracellular and intracellular sequestration of accumulated Cd(II). The wall became relatively thicker (500 nm) and was lined with an extracellular fibrillar material (Fig. 3c), indicating the presence of polysaccharide molecules. The precipitation outside the cell seemed to be the first defense of *A. awamori* against Cd(II) toxicity. Decreasing the bioavailability of the toxic metals/metalloids can occur through extracellular complexation, precipitation and binding to cell wall constituents (Pòesi 2011). Also, the Cd(II)-loaded cells showed an undulating plasma membrane generating vesicle. In addition, giant and distorted mitochondria were observed (Fig. 3d). This retraction may suggest the loss of plasma membrane turgidity and may be related to the exocytosis to the periplasmic space (Osumi 1998). This distortion may be due to the oxidative stress of Cd(II). Turrens (2003) demonstrated that despite the presence of various production sites throughout the cell, mitochondria appear to be the main intracellular source of ROS. Septa tripled their thickness (670 nm) thanks to the deposition of amorphous masses that first became evident in the transverse wall. Dark precipitates were very clear within the cell wall fibrilla of the septum (Fig. 3d). Also, vacuolation was very evident. Vacuoles were outlined and filled with electron-dense deposits (Fig. 3e). Vacuolation and compartmention are essential for metal detoxification (Gonzalez et al. 2008). In some Cd(II)-loaded cells, there was a complete lyses to the internal organelles which were substituted by many electron-dense granules (Fig. 3f). Nies (1999) demonstrated that the toxic effects of Cd(II) may be summed up under the general headings thiol-binding and protein denaturation, interaction with calcium metabolism and membrane damage, interaction with zinc metabolism, or loss of a protective function.

POD, PPO and GR

Jaeckel et al. (2005) reported that the modulation of the antioxidant status is an important adaptive response to heavy metals. Antioxidant enzymes have been thought of as the first line of defense in response to oxidative stress. The activities of POD, PPO and GR were measured to elucidate their role in coping with oxidative stress induced by Cd(II) exposure. Activity of the studied antioxidant enzymes in the mycelia of *A. awamori* gently decreased by treatment with different concentrations of Cd(II) (Fig. 4). At 200 mg/l concentration of Cd(II), a complete inhibition in PPO and GR activities was observed. The gradual decrease of enzyme activity suggests that the generated ROS caused irreversible oxidative damage in the fungal cell and inhibit POD activity (Guelfi et al. 2003). Hossain and Kermasha (1998) mentioned that metallothioneins bind selectively large amounts of heavy metal ions such as Zn(II), Cu(II), Cd(II) and Hg(II) and act as inhibitors of PPO activity. In general, GR is a FAD-containing protein that reduces oxidized glutathione to glutathione

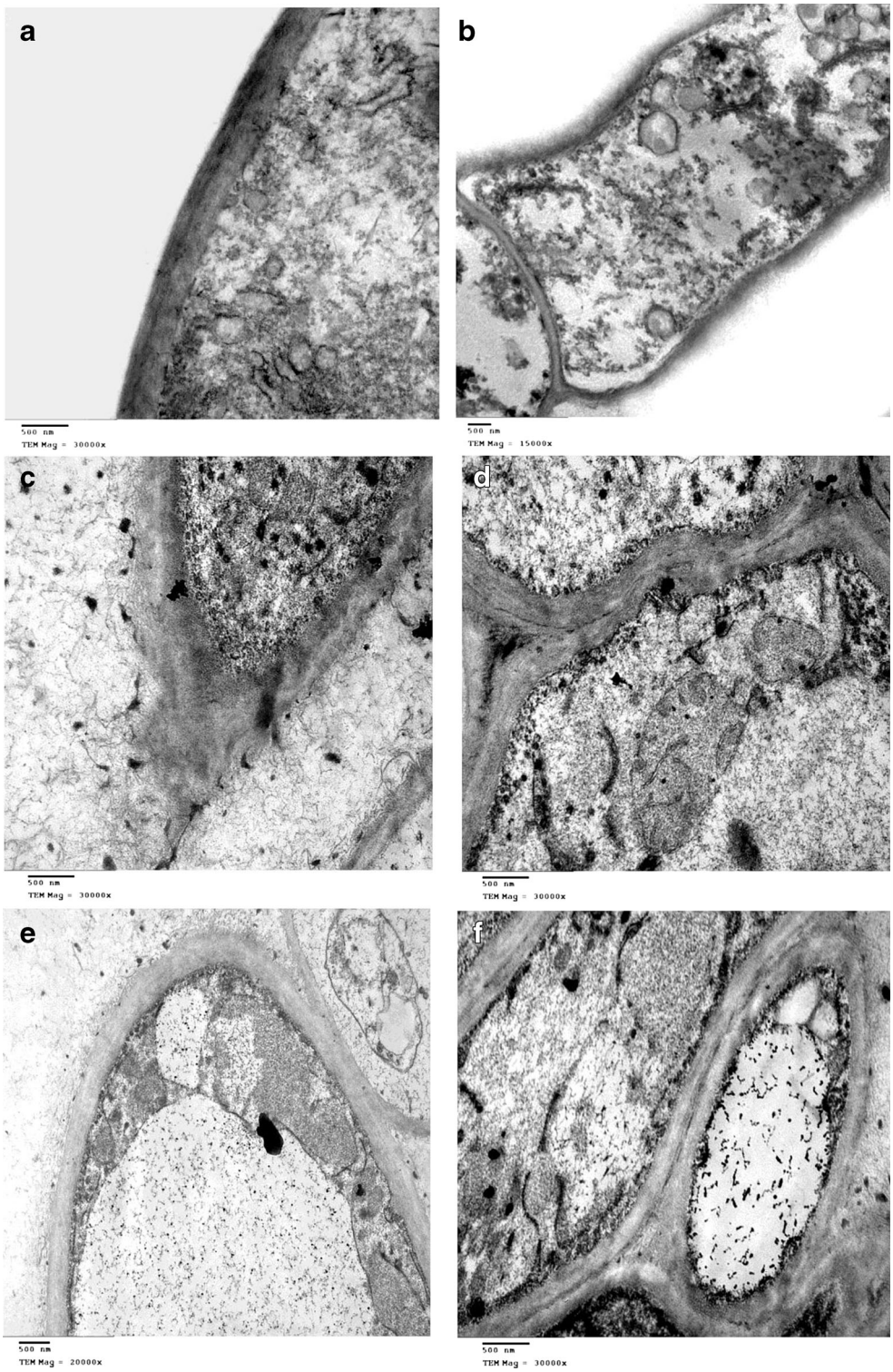

Fig. 3 TEM *A. awamori* (**a** and **b**) native cells and (**c**–**f**) Cd(II)-stressed cells

using NADPH as an electron donor (Sato et al. 2009), but it does not appear to be essential to the ability to adapt to oxidative stress (Li et al. 2009). Cadmium and some other metals caused an inhibition of antioxidative enzymes, especially of GR (Schützendübel and Polle 2002).

Total antioxidants

Antioxidants (both enzymatic and nonenzymatic) provide a protection against deleterious metal-mediated free radical attacks (Volka et al. 2013). Thus, the influence of Cd(II) on enzymatic antioxidant production was determined (data not shown). The

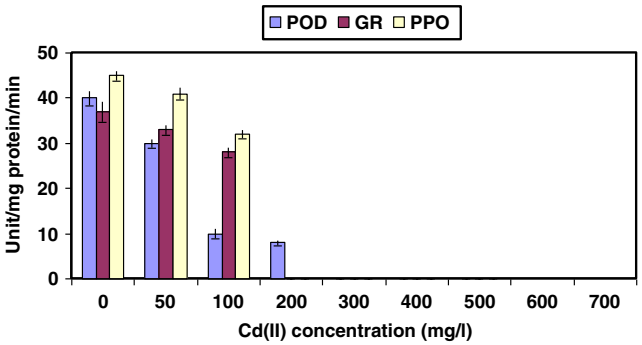

Fig. 4 Peroxidase (POD), polyphenol oxidase (PPO) and glutathiductase (GR) activity in *A. awamori* exposed to different Cd(II) concentrations

antioxidant concentration greatly increased (571.5 μM) after exposure to the lowest Cd(II) concentration (50 mg/l). This was followed by a sudden decrease (214.5 μM) at 100 mg/l concentration of Cd(II). Cadmium(II) induced a severe oxidative stress and more antioxidants were produced. At high concentrations of Cd(II), it seems that the fungus cannot cope with a severe Cd(II) toxicity. However, it is apparent that total antioxidant level had a big role in alleviating stress induced by Cd(II).

Effect of Cd(II) on soluble protein content *of A. awamori*

Among the mechanisms of heavy metal tolerance, proteins are considered to be one of the most important chelators in the bioavailability reduction of heavy metals. In addition to chelation, proteins can act as enzymes to ameliorate metal toxicity (Weishuang et al. 2009). Changes in the intracellular and extracellular soluble protein content in *A. awamori* under different concentrations of Cd(II) were studied (Fig. 2S). Intracellular and extracellular protein content increased initially with Cd(II) treatments and reached the maximum values at 100 mg/l concentration of Cd(II) (26.8 % and 243.9 % increase, respectively, compared with to control). *A. awamori* synthesized more protein up to a certain level of Cd(II) treatment for the survival strategy which again may reflect the possibility of the Arndt-Schulz effect. This results in the accumulation of toxins in non-lethal concentrations at the cell surface to cause alteration in the cellular permeability. This in turn leads to freer flow of nutrients within cells and thus metabolic activity increases (Babich and Stotzky 1980; Ahonen-Jounarth et al. 2004). A decrease in intracellular protein content at 300 mg/l Cd(II) may be a result of intolerance to that high of a Cd(II) concentration. Guelfi et al. (2003) suggested that the higher concentrations of Cd(II) induced the autolysis of the *A. nidulans* mycelium, with subsequent proteolytic breakdown and reduction in the protein content.

Effect of Cd(II) on thiol content of *A. awamori*

Thiol compounds are essential components of Cd(II) detoxification pathways in various organisms (Hall 2002). Intracellular thiol content increased gradually with an increase in Cd(II) stress during the growth of the fungus in liquid medium (Fig. 3S). The fungus synthesized the maximum amount of intracellular and extracellular thiols at 300 mg/l concentration of Cd(II) (253.5 % and 138.1 % with respect to control, respectively). The level of intracellular thiol content was found to be elevated during metal stress (Schmoger et al. 2000). This is because thiols are well-known for metal chelation and detoxification. Some important members of the thiol family are capable of binding heavy metal ions via thiolate coordination in fungi (Bellion et al. 2007).

Oxalic acid secretion

The production of organic acids by fungi has profound implications for metal speciation, physiology and biogeochemical cycles. The formation of toxic metal oxalates may confer tolerance and ensure survival in contaminated environments (Gadd 1999). HPLC chromatograms of control and Cd(II)-stressed samples (Fig. 5a and b, respectively) revealed that the concentrations of oxalic acid were found to be 16.1 and 19.8 mg/ml, respectively. These results indicated that Cd(II) stress stimulated the production of oxalic acid (23.8 %, with respect to control). The detoxification strategies of *A. foetidus* occurred by the production of extracellular metabolites (e.g. citrate, oxalate) that are capable of adsorbing and precipitating the metal ions on the cell surface (Ge et al. 2011).

Biosorption results

Initial metal ion concentration

As the metal ion concentration increased, biosorption capacity also increased and reached saturation values of 320 and 200 mg/g Cd(II) for live and dead biomass, respectively, which were achieved at 500 mg/l concentration of metal ions (Fig. 4S). The uptake capacity was reduced with the rise in initial metal ion concentration to 700 mg/l. At lower initial solute concentration, the ratio of the initial moles solute to the available surface area was low; subsequently, the fractional sorption becomes independent of the initial concentration. When the surface active sites have been completely covered with metal ions, the adsorption has reached a limit which can be described by the maximum biosorption capacity. At higher concentrations, the sites available for sorption become fewer compared to the moles of solute present and, hence, the removal of solute was strongly dependent upon the initial solute concentrations (Binupriya et al. 2007).

The biosorption capacity of *A. awamori* was high for Cd(II), although that strain was sensitive for Cd(II). In this respect, Ruta et al. (2010) reported that the most of tolerant strains of yeast were hypo-accumulators, owing to their

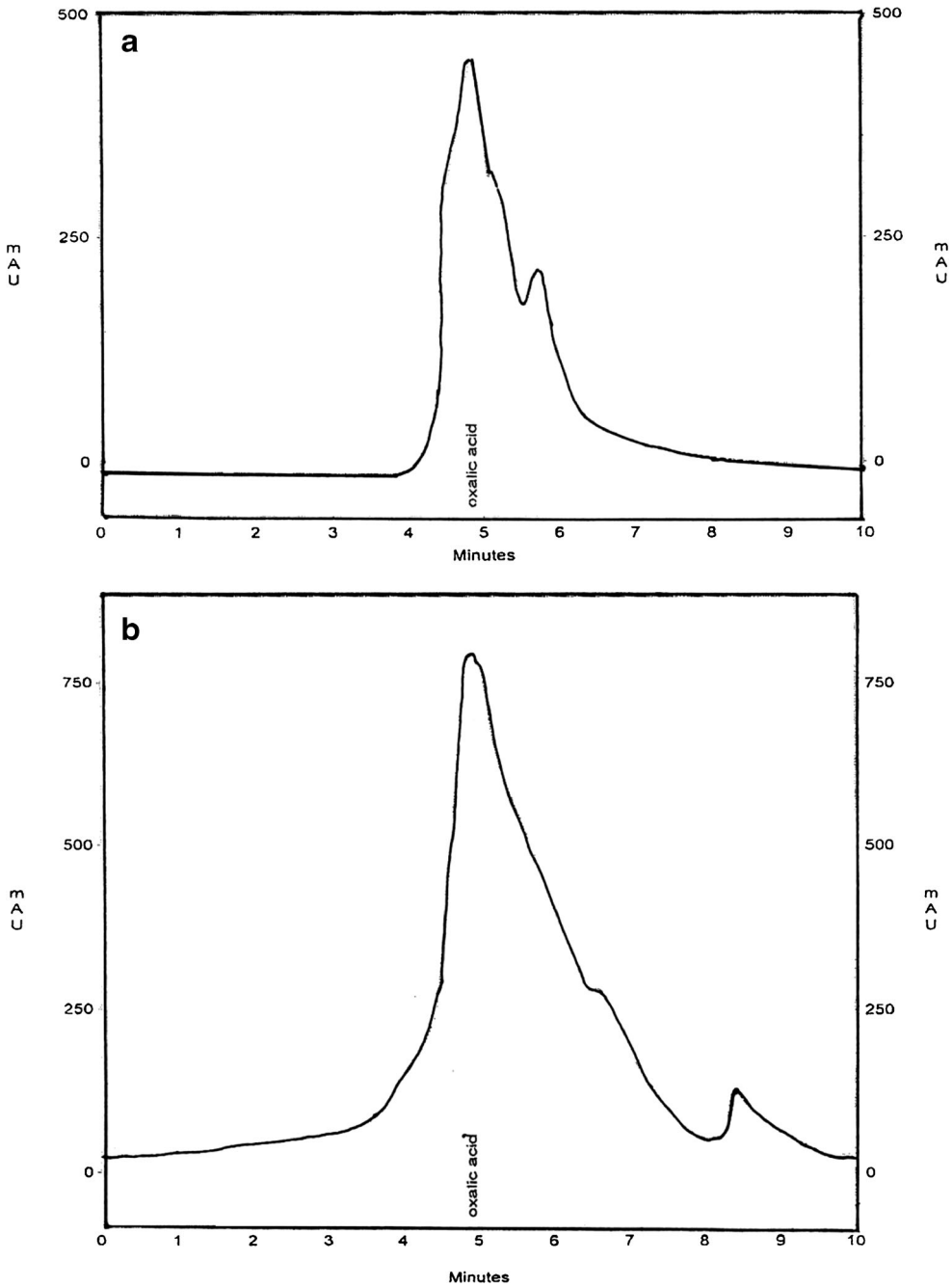

Fig. 5 HPLC chromatograms of *A. awamori* (**a**) native cells and (**b**) Cd(II)-stressed cells

gained tolerance to either reduced metal uptake or to enhanced export activity. Another explanation by Viraraghavan and Srinivasan (2011) was that the biosorption was higher for metals with larger ionic radius, the exceptions being chromium and the alkali metal ions. The ionic radius of Cd(II)is 151 pm.

Initial pH

The effect of solution pH on the biosorption process can vary with the type of biomass and the type of metal ion being studied (Akar et al. 2007). Solution pH influences surface metal binding sites of the biosorbents and the chemistry of the cell wall as well as physicochemistry and hydrolysis of metals (Tsekova et al. 2010). The effect of initial pH was evaluated in the range of 2–6 to avoid the precipitation of metal hydroxide. Yahya et al. (2009) and Xiao et al. (2010) stated that, at a solution pH of above 6.3, the metal ions precipitate making biosorption impossible. Low pH (2.0) had a more drastic effect on Cd(II) biosorption capacity of live cells (23.0 mg/g) than that of dead cells (45.0 mg/g) (Fig. 5S). This is due to protein denaturation at low pH (Rothschild and Mancinelli 2001). The Cd(II) removal capacities of dead and live cells increased very sharply with an increase in pH to

5.0. A sudden increase in sorption with a slight increase in pH is often referred to as an adsorption edge (Varshney et al. 2011). The increase in biosorption capacity may be related to the ionization of functional groups which serve as the binding sites. In this study, the lower biosorption capacity at pH values below 4.0 may be due to hydrogen ions that compete with metal ions on the biosoprtion sites. According to Rathinam et al. (2010), the enhancement of biosorption capacity in the range of pH 3.0–5.0 was due to more negatively charged functional groups (carboxyl, amine or hydroxyl) being exposed. The decrease in biosorption above pH 5.0 might be attributed to the speciation of the metal ions, such as the formation of $Cd(OH)_2$ ions that do not adsorb well, and resulted in the reduction of biosorption.

Biosorbent concentration

The biosorption of Cd(II) with different biosorbent concentrations was studied. The uptake capacities of live and dead biomass for Cd(II) decreased with increasing biomass concentration (Fig. 6S). The highest uptake capacities of live and dead biomass for Cd(II) were observed at 1 g/l concentration. At a given equilibrium concentration, the biomass takes up more metal ions at lower than at higher cell densities (Mehta and Gaur 2005). It has been suggested that electrostatic interactions between cells can be a significant factor in the relationship between biomass concentration and metal sorption. The biosorption capacity decreased from 322 and 200 to 82.0 and 53 mg/g for live and dead biomass, respectively, as the biomass concentration increased from 1 to 5 g/l. In this connection Wang and Chen (2006) reported that at a given metal concentration, the lower the biomass concentration in suspension, the higher will be the metal/biosorbent ratio and the metal retained by sorbent unit, unless the biomass reaches saturation. They added that high biomass concentrations can exert a shell effect protecting the active sites from being occupied by metal. The result of this is a lower specific metal uptake.

Contact time

It can be seen that the primary fast phase of biosorption by dead biomass in the case of Cd(II) was within the first 2 h and this was followed by a second slow phase until equilibrium (Fig. 7S). A similar trend was observed by Wang et al. (2010). On the contrary, the rate of Cd(II) biosorption by live biomass was slow and reached equilibrium within 6 h. Metal ion uptake is known to involve an initial rapid phase (passive uptake), followed by a much slower phase (active phase). The first stage is physical adsorption or ion exchange at the surface of the biomass and accounted for the major part in total metal uptake, while the second one contributed to a smaller part (Goyal et al. 2003).

FTIR spectroscopy

One of the major challenges in knowing the chemical groups involved in biosorption is the complex nature of the microbial biosorbent material. The cell wall polymers provide a multitude of chemical groups such as hydroxyl, carbonyl, carboxyl, sulfhydryl, thioether, sulfonate, amine, imine, amide, imidazole, phosphonate and phosphodiester (Gupta and Mohapatra 2003). Volesky (1987) studied the chemical composition of the walls of *A. niger* and reported (%) protein 8.3; chitin 8.3; chitosan 18.3; mannan 45.5; phosphate 11.5; and carbohydrate and lipids 8.1.

The FTIR spectra were taken to obtain information on the nature of possible cell-metal ion interactions of metal-unloaded and -loaded biomass in the range of 40,000–400 cm^{-1} is shown in Fig. 6a–c. There are some guide peaks in the spectra of the control which are helpful for achieving this goal (Fig. 6a). The position or the intensities of these peaks are expected to be changed upon of the interaction of biomass with Cd(II), and also new peaks and shoulders are found in the spectra of Cd(II)-loaded live and dead samples (Fig. 6b and c, respectively) which gave an indication on chelation between oxygen-, nitrogen-, sulphur-, or phosphorus-containing ligands of biomass with metal ions. There was a marked shift in the wave number from 3,402 to 3,410 and 3,145 cm^{-1} in live and dead Cd(II)-loaded biomass, respectively, indicating the interaction of an $–NH_2$ asymmetric stretch mode of amines and –OH groups with Cd(II) uptake. The changes in the intensities of peaks at 2,926.5 cm^{-1} may be because of the interaction of –CH stretching vibrations of $–CH_2–CH_2$. The appearance of shoulders at wave number 2,874 cm^{-1} in the case of live Cd(II)-loaded biomass and 2,853 cm^{-1} in the case of dead Cd(II)-loaded biomass may be because of the interaction of –CH stretching vibrations of CH_2 $–CH_3$. The appearance of peaks at wave number 2,164.7 cm^{-1} and 2,156 cm^{-1} in live and dead Cd(II)-loaded biomass can be assigned to C=O and C=N groups. A small shift of bands at 1,637 cm^{-1} with a significant increase in the intensity after metal ions uptake (in the case of live Cd(II)-loaded biomass) can be attributed to a CO stretching mode conjugated to a NH deformation mode and amide I band. A shift of band at 1,550 cm^{-1} with an increase in the intensity was more evident in live and dead Cd(II)-loaded biomass. This was the result of an amide II and NH deformation mode conjugated to a C=N deformation mode. The marked shift at 1,424 cm^{-1} for live Cd(II)-loaded biomass was indicative of sulfonyl and sulfonamide groups. A small shift of bands at 1,424 cm^{-1} was observed after Cd(II) uptake by dead biomass. The role of sulfonyl, sulfonamide groups and amide III can be observed again in the appearance of peaks in the wave number from 1,372 to 1,377 cm^{-1} after Cd(II) uptake by live and dead biomass. FTIR spectra of live biomass incurred more changes than that of the dead one, indicating that more functional

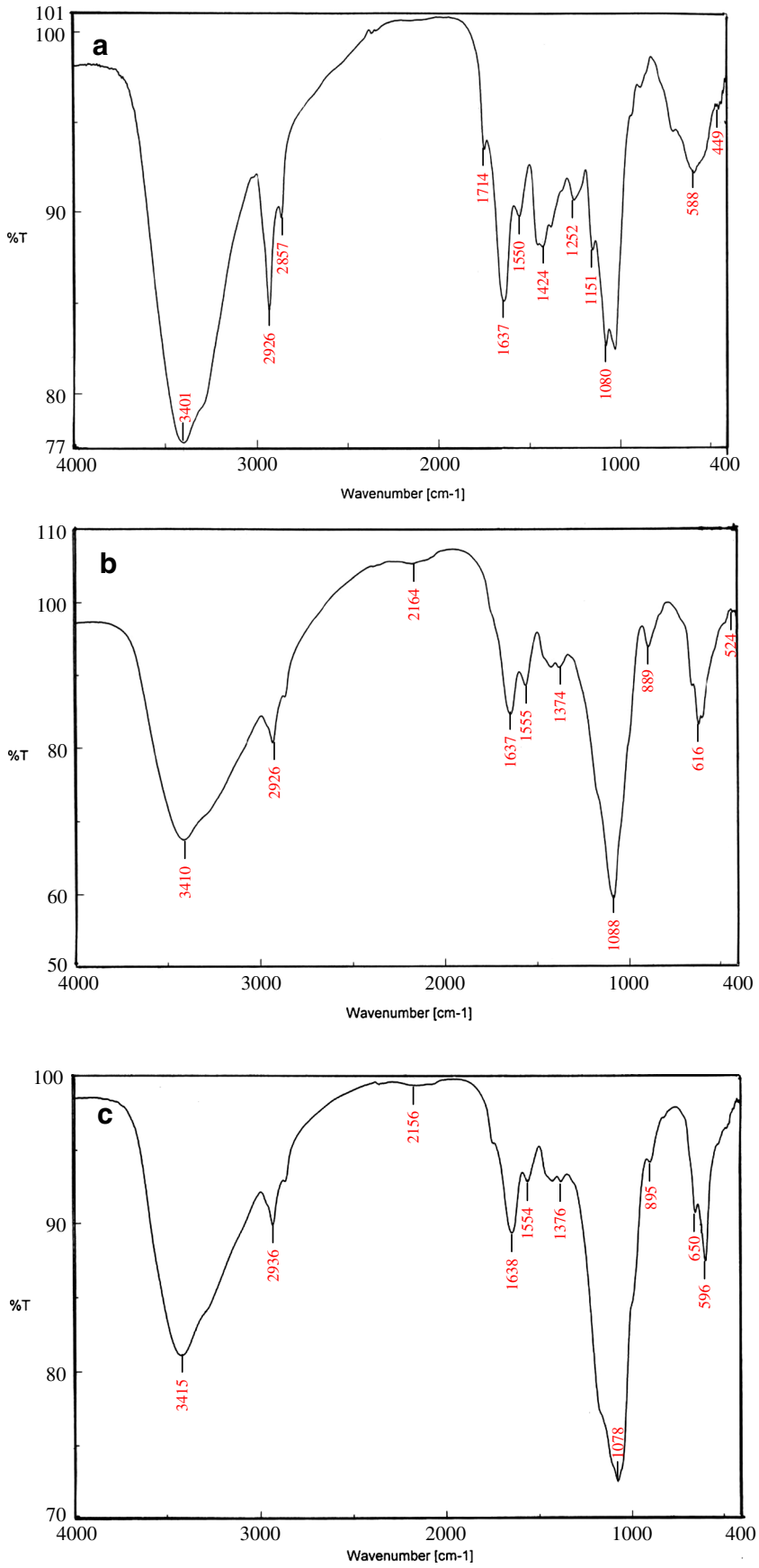

Fig. 6 FTIR spectra of *A. awamori* (**a**), native cells, (**b**) Cd(II)-loaded live cells and (**c**) Cd(II)-loaded dead cells

groups were involved in the biosorption process. The disappearance of bands located at 1,153 and 1,151.3 cm^{-1} which are attributed to the loading of Cd(II) was explained by the involvement of S=O stretching. Shifting of bands at 1,081 cm^{-1} coupled with a sharp or significant decrease in the intensity and disappearance of bands at 1,030.8 cm^{-1} may be due to the interaction of adsorbed metals with sulfoxides, S=O stretching, sulfones, sulfonic acids and sulfonamides by live and dead biomass. Appearance of new bands after Cd(II) uptake by live and dead biomass at 890 and 896 cm^{-1}, respectively, indicated the intervention of phosphorus and P=S stretching in the process. The appearance of new bands at wave numbers ranging from 616.2 to 673.3 cm^{-1} and a marked shift at 588.2 cm^{-1} ranging from after uptake of metal ions is indicative of C-S stretching. The role of C-S stretching appeared again in either marked shift or disappearance of band at 449.3 cm^{-1} after metal ions uptake. Soft metals, such as Hg(II), Cd (II) and Pb(II) form stable bonds with nitrogen-, sulphur-containing (soft) ligands CN^-, $R\text{-}S^-$, SH^-, NH_2^- and imidazole (Wang and Chen 2006). The greater the covalent index ($X^2_m r$) (where X_m is electronegativity and r is the ionic radius), the greater its potential to form covalent bands with

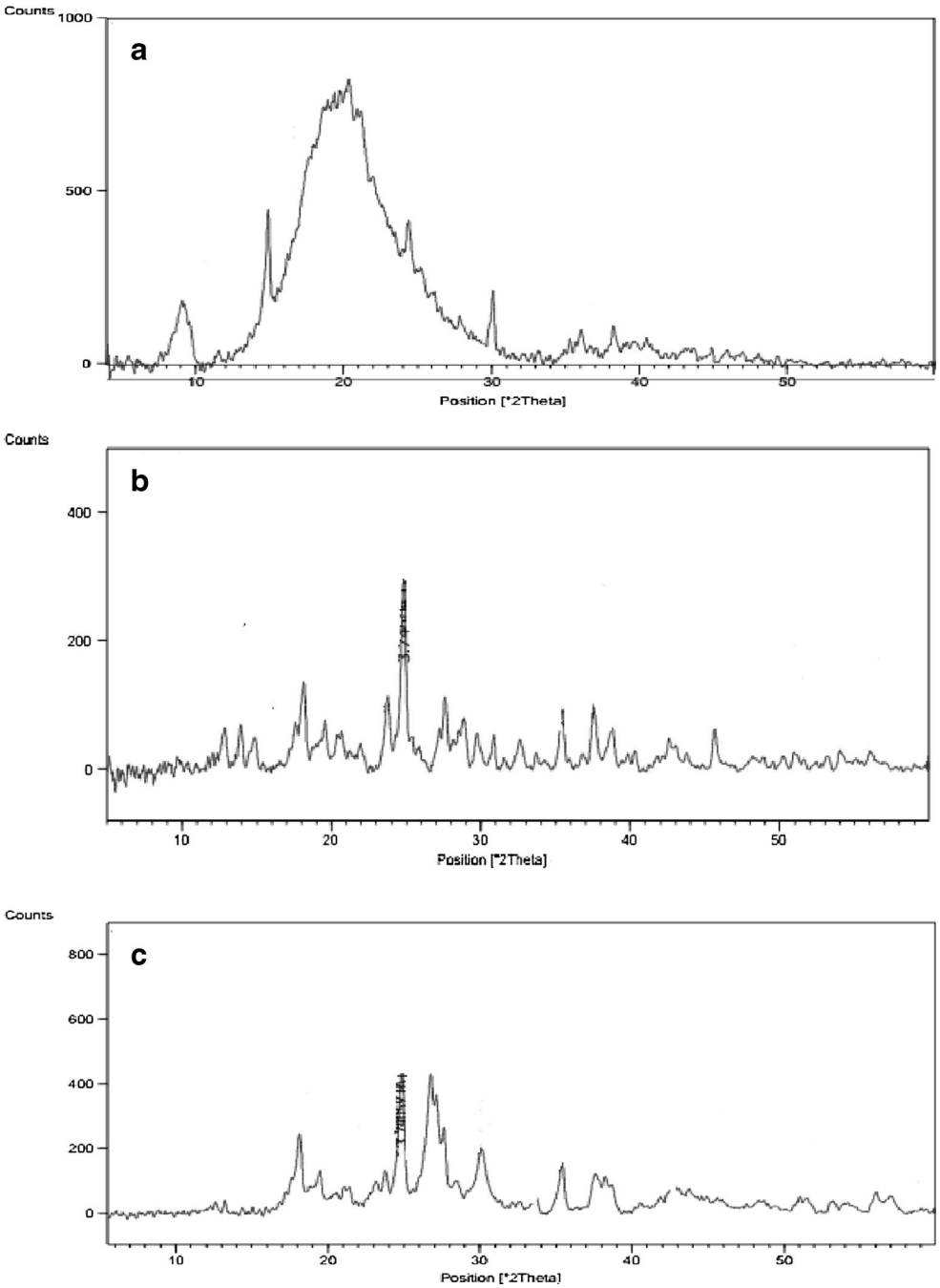

Fig. 7 XRD analysis of *A. awamori* (**a**), native cells, (**b**) Cd(II)-loaded live cells and (**c**) Cd(II)-loaded dead cells

biological ligands, generally in order S>N>0 (Chen and Wang 2007). The covalent index of Cd(II) is 2.71 (Brady and Tobin 1995). Li et al. (2013) reported that the FTIR analysis of *Aspergillus* sp.J2 indicated that the sulfur compound was involved in Se(IV) biosorption.

X-ray diffraction (XRD) analysis

To elucidate the chemical nature of *A. awamori* cell-bound Cd(II), a test biomass was subjected to XRD analysis before (control) and after Cd(II) sequestration (Fig. 7a–c). In contrast to untreated (control) biomass, which is expectedly amorphous (Fig. 7a), XRD spectra for live and dead biomass loaded with Cd(II) showed distinct, reproducible patterns typical for the presence of crystalline materials. Following Cd(II), it was noted that peaks in live biomass were more pronounced than those of the dead one. After scanning by X-ray over Cd(II)-loaded live biomass, Fig. 7b showed 36 peaks at 2θ ranging from 4.42 to 59.04 A° and corresponding to respective d-spacing ranging from 20.00 to 1.56 A°. On the other hand, the XRD pattern of Cd(II)-loaded dead biomass (Fig. 7c) showed 27 peaks at 2θ ranging from 18.13 to 58.21 A° and corresponding to respective d-spacing from 4.89 to 1.58A, d-spacing from 4.87 to 1.56 A°. Based on spacing d-values, these peaks are attributed to the presence of crystalline Cd(II) sulphate hydrate; $CdSO_4 \cdot H_2O$. FTIR spectroscopic analysis of the tested biomass also confirmed the involvement of sulfur-oxygen compounds in these metal ions uptake.

Energy dispersive X-ray (EDX) microanalysis

EDX analysis is based on the principle that X-rays can be absorbed by matter, which gives rise to X-ray absorption spectra. These X-ray dispersion spectra may be detected at

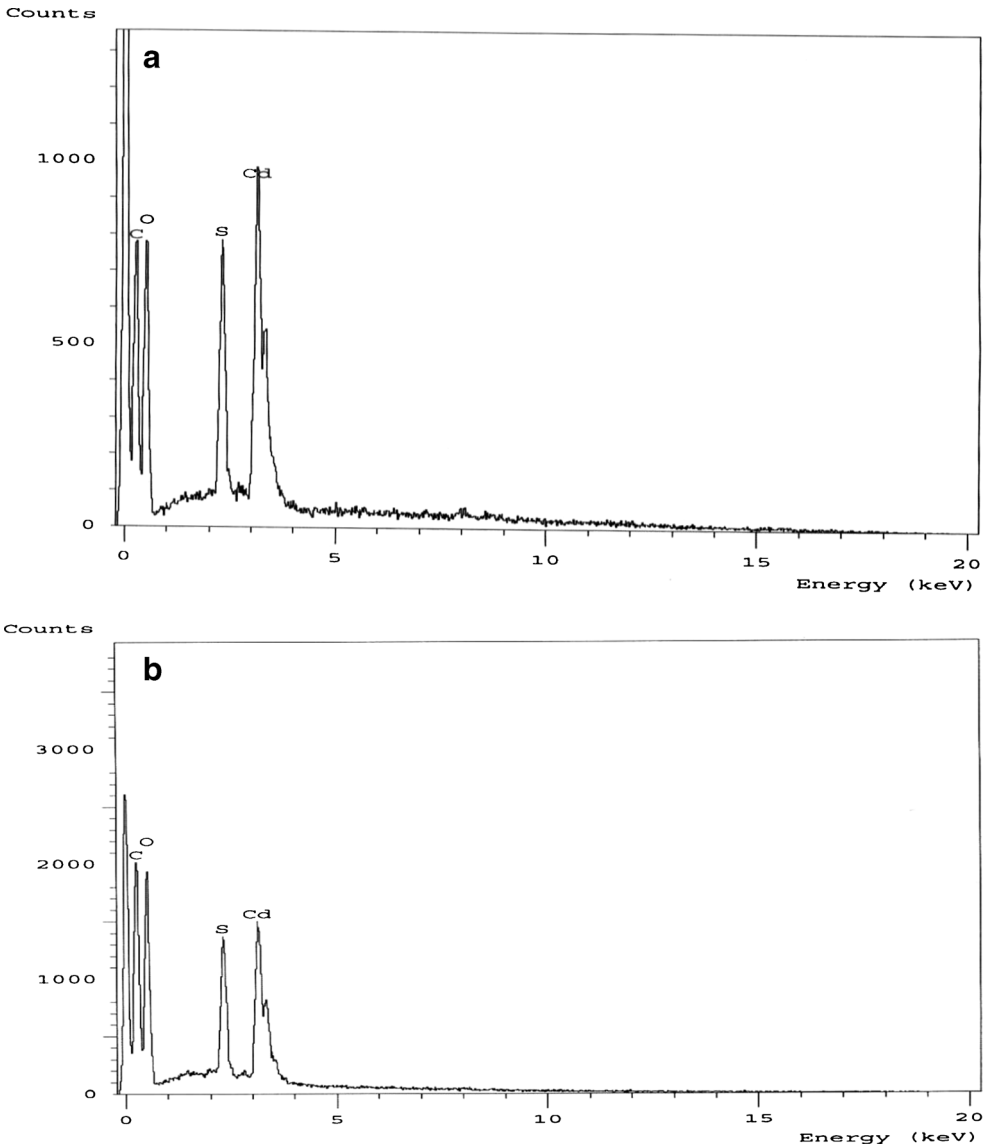

Fig. 8 EDX microanalysis of *A. awamori* (**a**) Cd(II)-loaded live cells and (**b**) Cd(II)-loaded dead cells

various angles that can then be correlated with the complex formed (Gupta and Mohapatra 2003). X-ray peaks showed a broadening in the peak full-width half-maximum (FWHM) which confirmed the proportion led by the size of pellets of $CdSO_4 \cdot H_2O$. Also, the height of the peak was proportional to the weight percent of the compounds or their ingredients Cd(II), S and O. EDX spectra and the concentrations of semi-quantified results of Cd(II)-loaded live and dead biomass were represented in Fig. 8a and b, respectively. This bulk technique gives an elemental ratio of the population as a whole and analyzes the whole pellet following the metal exposure. EDX spectra of Cd(II)-loaded live and dead biomass showed distinct peaks for oxygen, sulfur and Cd(II) with element % 43.7, 6.1, 50.2, 67.02, 6.99 and 25.99, respectively. EDX showed an excellent agreement with the corresponding FTIR and XRD analyses. FTIR indicated the involvement of cellular sulfur-oxygen compounds in both metal ions binding. XRD analysis confirmed the presence of $CdSO_4 \cdot H_2O$ on live and dead biomass.

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