Egypt. J.Microbiol. **48**, pp. 23-52 (2013)

An Investigation on Tolerance and Biosorption Potential of *Aspergillus awamori* ZU JQ 965830.1 to Cu(II)

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THE MINIMUM inhibitory concentration (MIC) value of *A.awamori* for Cu(II) was 3100mg/l and a complete inhibition of biomass production was observed at 800mg/l concentration of Cu(II). Slight changes were observed by SEM investigation in Cu(II)-stressed biomass. Quantification of Cu(II) was performed by EDAX. Transmission electron microscopy investigation (TEM) confirmed the involvement of extracellular adsorption, intracellular penetration through the cell wall and vacuolation. Cu(II) stress induced noticeable changes in the activities of polyphenol oxidase(PPO), glutathione reductase (GR) and peroxidase (POD) and in the concentration of total antioxidant, soluble protein and thiol. High performance liquid chromatography analysis (HPLC) revealed that Cu(II) stress stimulated the production of oxalic acid .Maximum Cu(II) uptake capacity was achieved at pH 4.0, initial metal ion concentration 500mg/l and biomass dosage 1g/l. Maximum Cu(II) uptake capacities were reached after 180 min for live biomass and 30 min for dead biomass. Fourier transform infrared spectroscopy (FTIR) results gave an indicateion of chelation between oxygen-, nitrogen-, phosphorus- and especially sulphur-containing ligands of biomass with metal ions. X-ray diffraction analysis (XRD) revealed the presence of $CuSO_4.H_2O$ in live and dead biomass. EDAX confirmed the occurrence of sulphur, oxygen and Cu(II) in the cell wall.

Keywords: *Aspergillus awamor;*, Cu(II), Stress, Antioxidant enzymes, Biosorption.

Several inorganic and organic compounds such as heavy metals, fuels and petroleum industry products cause soil and water contamination. For this reason, research focused toward better decontamination methods and the development of new technologies is imperative (Velásquez & Dussan, 2009).

Remediation methods for heavy metals differ from those for organic compounds. As compared to organic compounds, metals are non-biodegradable (Gupta & Rastogi, 2008). Therefore, biomobilization is a valid concept in the management of metal pollution. Some metal ions, like Cu(II) and Zn(II) are essential for biological processes, but other nonessential metals like Cd(II) and Hg(II) are considered highly toxic elements to nearly all organisms even at low

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concentrations (Guelfi *et al.,* 2003). However, Cu(II) is toxic to most organisms at elevated concentrations, largely through enzyme inhibition and oxidation of membrane components as a result of the ability of Cu(II) to generate toxic hydroxyl radicals (Melo *et al.,* 2004). Membrane damage then leads to rapid leakage of ions and other low molecular weight compounds (Soares *et al.,* 2003).

Various techniques such as precipitation, coagulation, ion exchange, reverse osmosis, evaporation, filtration, electrochemical treatment and oxidation and reduction have been employed to remove toxic heavy metals from industrial wastewaters. Most of these techniques are ineffective or extremely expensive in terms of energy and reagent consumption, especially when concentrations of dissolved metals are on the order of 1-100mg/l (Tang et *al.,* 2008). Of these techniques, biosorption using the biomass of live or dead microorganisms has been proven effective as a consequence of high surface-to-volume ratio and selective adsorption of heavy metal ions. Additionally they can be used over a broad range of environmental conditions, reactions are fast and reversible are eco-friendly, economic and give excellent performance (Kapoor *et al.,* 1999; Issac *et al.,* 2012 and Huang *et al.,* 2013). Bioaccumulation and biosorption are two processes, one based on the assimilation of metals inside the biomass and another process is biosorption, in which metallic ions remain at the cell surface (Vijayaraghavan & Yun, 2008). Fungi have a high percentage of cell wall material that shows excellent metal-binding properties and exhibits marked tolerance towards metals and other factors such as low pH (Zafar *et al.,* 2007). Recently, heavy metal accumulating strains have found a new role as small factories for the production of nanoparticles (Klaus-Joerger *et al.,* 2001). In the concept of biosorption, several chemical processes may be involved, such as adsorption, ion exchange and covalent bonding with the biosorption sites of the microorganisms including carboxyl, hydroxyl, sulfhydryl, amino and phosphate groups (Montazer-Rahmati *et al.,* 2011). The status of biomass (live or dead), types of biomaterials, 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 described according to the location where the metal is removed from the solution extracellular accumulation/ precipitation, cell surface sorption/ precipitation or intracellular accumulation (Veglio & Beolchini, 1997).

Fungi such as *Rhizopus arrhizus* (Bahadir *et al.,* 2007), *A. niger* (Tsekova *et al.* 2010 and Kumar *et al.,* 2012), *A. awamori* (Gochev *et al.,* 2010 and Velkova *et al.,* 2012) have been used to remove different heavy metals.

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

Material and Methods

Microorganism, growth conditions and preparation of biosorbent

Aspergillus awamori ZU JQ 695830.1 was obtained from our laboratory stock cultures (Mycology Lab., Botany Department, Faculty of Science, Zagazig University). The isolate was cultured on potato/ glucose agar slants (PDA) (Gams *et al.,* 1998) and stored at 4^{o}C.

For the preparation of the biosorbent, *A. awamori* was cultured on potato/ glucose broth (PD). After 5 days of incubation at 3^{o}C on a rotary shaker at 125rpm, 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 6^{o}C and powdered to be used as a dead biomass in the biosorption experiments.

Metal solutions

Stock metal solutions of Cu(II) were prepared by dissolving appropriate quantities of $CuSO_4.4H_2O$ 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 $CuSO_4.4H_2O$ was aseptically added to the sterilized PDA medium to final concentrations ranging from 0 to 3200g/ml. The plates were centrally inoculated with 5mm fungal plugs from 4 days old fungal colonies in three replicates and were incubated at 3^{o}C for 7 days. MIC was identified as the minimum concentration of Cu(II) that inhibited visible growth of *A. awamori*.

Effect of Cu(II) stress on A. awamori growth

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

To study the effect of Cu(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 250ml Erlenmeyer flasks containing 50ml of PD. Sterilized solutions of Cu(II) sulphate were aseptically added to the sterilized PDA medium to get final concentration ranging from 0-1000µg/ml, then incubated at 30^{o}C for 6 days on a rotary shaker at 125rpm. The biomass was harvested and dried to a constant weight at 60^{o}C.

Scanning electron microscopy analysis (SEM)

*Aspergillus awamo*ri cells were fixed in 2.5% glutaraldehyde at 4^{o}C, for 24h and then post-fixed in 1.0% osmium tetraoxide at room temperature for 1h (Harely & Ferguson, 1990). The samples were then dehydrated in acetone and coated with gold. The scanning and photographing were done using a Jeol scanning electron microscope (JEM-1200XII) (Regional Center of Mycology and Biotechnology, Cairo, Egypt).

Energy dispersive X- ray microanalysis (EDAX)

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

Transmission electron microscopy analysis (TEM)

A. awamori cells were fixed in 2.5% glutaraldehyde for 3h (Gupta & Berridge, 1966), washed twice with 0.2M phosphate buffer of pH 7.4 for 30min, then post-fixed in 1.0% osmium tetraoxide for 2h (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^{o}C. Samples were dehydrated in a graded ethanol series (50%-100%). They were then 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 (ultramicrotome RMC USA) into ultrathin section about 70nm. These ultrathin sections were constructed with uranyl acetate (Sptempack & Ward, 1969) followed by lead citrate (Reynolds, 1963) each for 30min. Transmission and photographing were done using a JEOL- 1010 electron microscope (Regional Center of Mycology and Biotechnology, Cairo, Egypt).

The response of A. awamori to Cu(II) stress

The effects of Cu(II) on the concentrations of total soluble protein, thiol content and total antioxidant and on the activities of peroxidase, glutathione reductase and polyphenol oxidase were determined. The fungal mycelia were ground using a cold mortar in an ice bath with 50mM cold phosphate buffer (pH 7.0) of 50mM EDTA. The cell suspensions were centrifuged at 6000rpm for 12min at 4^{o}C. The supernatants and filtrates were used to elucidate the tolerance mechanism of *A. awamori* to Cu(II).

Polyphenol oxidase

Polyphenol oxidase (PPO) activity was determined according to Bergmeyer *et al.* (1974) with slight modifications. Briefly, the reaction mixture contained 200µl of enzyme preparation in 0.1M potassium phosphate buffer (pH 7.0), 0.2mM guaiacol, 5U/ml horseradish peroxidase and 10 mM catechol as substrate, in a total volume of 1ml. The reaction was incubated for 60 min at 30°C. After the reaction was frozen for 10 min, the developed color was measured at 436nm. One unit of the enzyme was expressed by the amount of enzyme that released 1µmol H_2O_2 per min under optimal assay conditions.

Glutathione reductase

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

Total antioxidant

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

Protein measurement

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

Assay of total thiol content

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

High performance liquid chromatography (HPLC)

To investigate the role of oxalic acid in the tolerance mechanism, analysis of oxalic acid in control and Cu(II)-stressed samples was carried out using HPLC system comprising of GBC UV/vis detector, GBC LC 1110 pump controlled by WinChrome chromatography ver 1.3 software. The eluent was 85% acetonitrile: 15% water and the column used was a Kromasil 100* 4.6mm with flow rate 1ml/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 Cu(II), batch biosorption experiments were carried out. In addition, a comparison between live (not growing) and dead biomass was studied.

All uptake experiments were performed by suspending the biosorbent in 100 ml of solution at the desired 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 initial pH on metal uptake, the pH of the solution was adjusted in the range between 2 to 6 *viz*. 2, 3, 4, 5 and 6 before mixing with biomass. The pH was adjusted to the required value with aqueous solution of 0.1N HCl or 0.1N NaOH. Initial Cu(II) concentration was 500mg/l, biosorbent dose was 1g/l at 28^{o}C and contact time was 30min (dead biomass) and 180 min (live biomass). The concentration of unadsorbed Cu(II) in the supernatants was measured by 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 Cu(II). Aliquots (50ml), of 150, 200, 300, 500 and 700 mg/l concentrations of Cu (II) were added to 1g/l biomass at 28^{o}C in 500 ml Erlenmeyer flasks.

Effect of biosorbent dose

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

Effect of contact time

The biomass concentration (1g/l) was exposed with 500mg/l Cu(II) solutions 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 24h 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:

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

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 ones were recorded over the region 400-4000 cm^{-1} with Perkin- Elmer 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 analysis (XRD)

X- ray diffraction patterns of powdered samples of metal-free and metal-loaded live and dead biomass were recorded in a Broker D8 Advanced target Cu

Koα powder diffractometer ($\lambda = 1.5418A^{o}$) over the range of 0-60 (2θ) (Central Metallurgical & Development Institute, Helwan, Egypt).

Results and Discussion

Heavy metal tolerance

Metal toxicity can be indicateed by MIC. Numerous methods have been employed to determine metal tolerance in fungi. *In vitro* assays include sensitivity to spore germination, mycelia growth extension and biomass production in the presence of various concentrations of metal salts in broth and/ or agar nutrient medium (Ahmad *et al.,* 2011). Results showed very high fungal tolerance to Cu(II) concentration up to 3100 mg/l which is more than 30000 fold the maximum admissible Cu(II) concentration in drinking water (0.1mg/l)(WHO, 2008). MIC has been reported to be species specific and metal dependant (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) for related fungus. They reported that MIC of Cu(II) of *A. niger* (GF-1), (GF-5) and (SF-5) were 318, 63.5 and 445mg/l, respectively. There are only three known mechanisms for heavy metal resistance (Nies, 1999). First, the accumulation of the respective ion can be diminished by efflux. Second, cations, especially the sulfer lovers (*e.g.* Cd(II)) can be segregated into complex compounds by thiol-containing molecules. Third, some metal ions may be reduced to a less toxic oxidation state.

Effect of Cu(II) on the growth of A. awamori

Figures 1a and b display the influence of Cu(II) on the fungal growth that was assessed in the terms of colony diameter and mycelia production over 6 days incubation. *Aspergillus awamori* was resistant to high concentrations of Cu(II). An increase in the length of the lag phase was evident only in Cu(II) concentrations starting from 1200 mg/l (Fig. 1a). A complete inhibition in the radial growth and biomass production was observed at 3100 mg/l and 800 mg/l concentrations of Cu(II), respectively. Growth reduction is a typical response of fungi to the toxicity of heavy metals (Baldrian, 2003). In 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). In liquid media, the pellets at 600 mg/l were larger and more yellowish in color than those of the control which were smaller and white. Filamentous fungi exhibit two extreme types of morphology in submerged culture, pelleted and filamentous forms (Li *et al.,* 2009). It was reported that when fungal cells suffer high oxygen concentration, they generally adopt morphological forms which reduce the surface area exposed to the environment, such as adhesion of mycelia, formation of small and smooth pellets, and increased pellet density so the inside cells are relatively resistant to the high level of oxygen minimizing ROS mediated damage (Bai *et al.,* 2004).

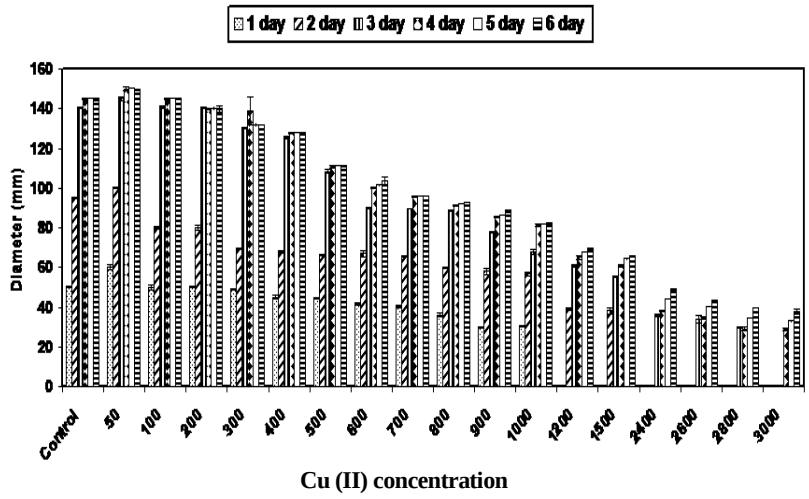

Fig. 1a. Effect of Cu(II) supplementation on the radial growth of *A. awamori*. Data represent the mean of 3 measurements

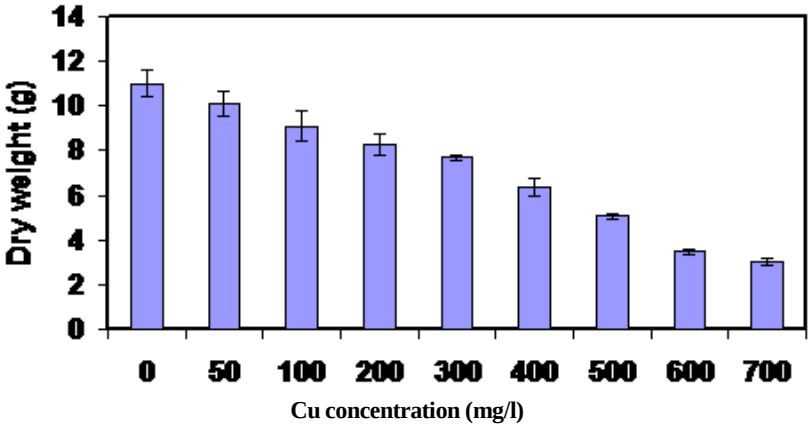

Fig. 1b. Effect of different concentrations of Cu(II) on mycelium dry weight of *A. awamori*. Data represent means of 3 measurements ± SE.

Assessment of *A. awamori* morphological changes in response to Cu(II) stress and the quantification of these metal ions within the fungus was performed by SEM investigation and EDAX microanalysis. Control biomass had heavily conidiated vesicles with smooth conidiophore (Fig. 2a) and normal conidia (Fig. 2b). Under Cu(II) stress, some granules appeared on the conidiophores. There were no sterigmata at the base of the vesicle. At the middle and the upper portion of the vesicle sterigmata appeared but without conidia (Fig. 2c). The inhibition of conidial formation may be due to the failure in either the division of sterigmata

nuclei or in the development of conidial initials. Some abnormal conidia were observed (Fig. 2d). 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 mycelial growth in saprophytic and mycorrhizal soil fungi (Ali, 2007).

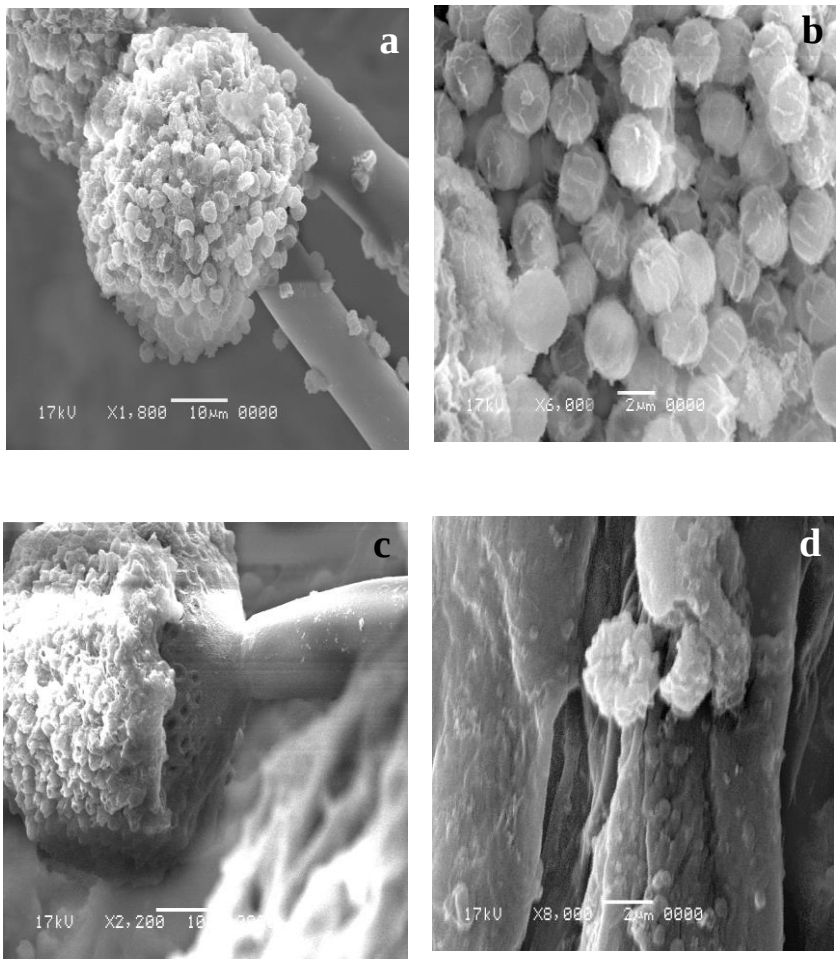

Fig. 2. **SEM *A. awamori* (a and b) native cells, (c and d) Cu(II)-stressed cells .**

EDAX microanalysis revealed that Cu(II) ions were bound to the fungal mycelia (Fig. 3). Quantification of Cu(II) ions gave confirmation of their accumulation by *A. awamori*.

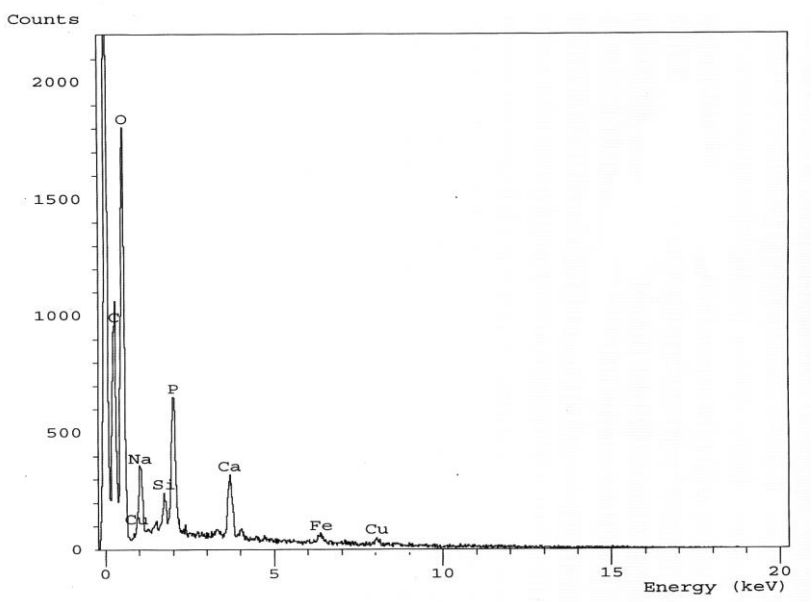

Fig. 3. EDAX of *A. awamori* Cu(II) – loaded cells.

Aspergillus awamori biomass samples before and after Cu(II) exposure were subjected to TEM investigation to ascertain the cellular localization of accumulated metals (Fig. 4a-d). Ultra thin sections of metal-less cells revealed a distinct cell wall 400nm in thickness, clear cytoplasm with normal mitochondria and few electron dense areas probably representing the genetic material and cytoplasmic deposits (Fig. 4a). Septum thickness was found to be 220 nm (Fig. 4b). From TEM micrographs of Cu(II)-loaded cells, it can be seen that cell walls of some cells became much thicker (710nm). Also, the cell wall seemed to be closely related with Cu(II) enrichment as Cu(II) particles are dotted within its layers and on the inner surface (Fig. 4c and d). Previous studies have indicateed that the fungal cell wall binds to approximately 50% of the metal ions (Joner *et al.*, 2000), most of which bind to negatively charged components of the cell wall, such as chitin and melanin (Ferrol *et al.,* 2009). Decreasing the bioavailability of the toxic metals/ metalloids can be managed through extracellular complexation, precipitation and binding to cell wall constituents (Pòesi, 2011). Vieira &Volesky (2000) reported that uranium sequestered in the layers of the cell wall material of *Rhizopus arrhizus* while thorium sequestered on the surface of the cell wall of the same fungus. Also, septa relatively doubled their thickness (500nm) (Fig. 4d) thanks to the deposition of amorphous masses that became evident in the transeverse wall. Plasmolysis and undulation of the plasma membrane were very evident. This was due to the toxicity of high concentration of Cu(II). This retraction may suggest the loss of plasma membrane turgidity and may be related to the exocyosis to the periplasmic space (Osumi, 1998). Electron dense deposits

were observed in the periplasm. Apart from cell wall biosorption, intracellular accumulation of Cu(II) occurred as well. Electron dense vacuoles were very apparent. Transporter proteins involved in metal tolerance facilitate the efflux of toxic metal ions from the cytosol or they allow metal sequestration into intracellular compartments, for example, vacuoles (Hall, 2002). Vacuolation and compartmention are essential for metal detoxification (Gonzalez *et al.,* 2008). Some fungi can precipitate metals in amorphous and crystalline forms, such as oxalates and other secondary mycogenic minerals (Burford *et al.,* 2006). Copper (II) tolerance by fungi has been extensively investigated, and the ability to prevent cellular entry or reducing accumulation of Cu(II) has been reported as the main mechanism for tolerance (Gadd & White, 1989).

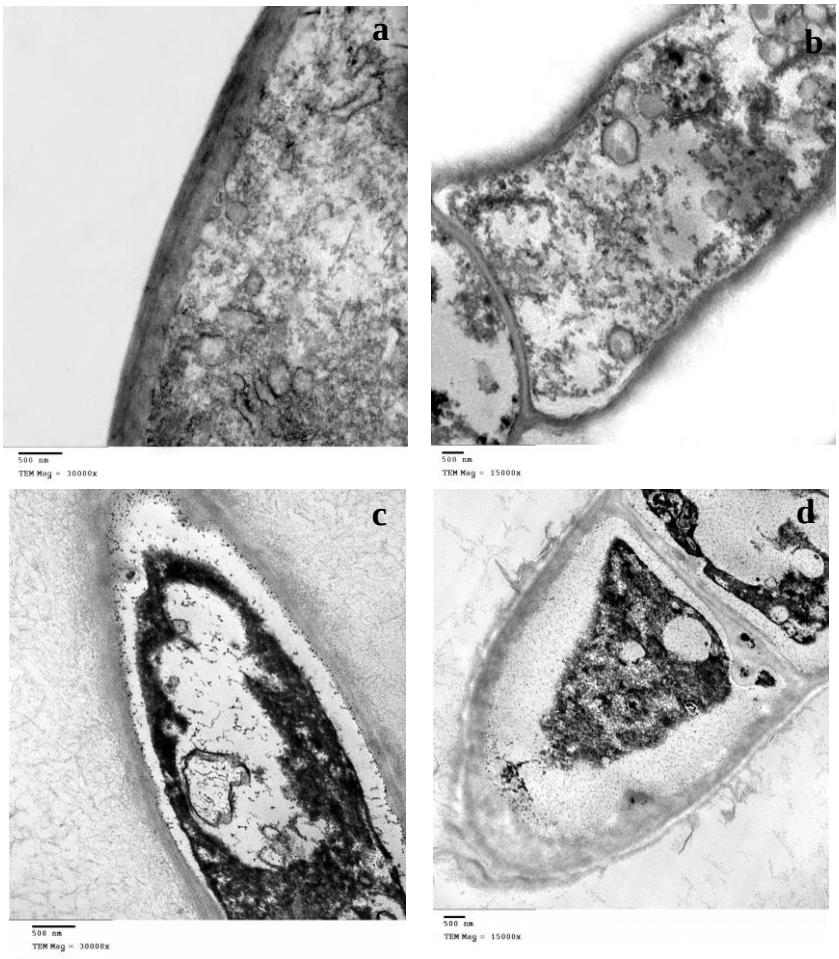

Fig. 4. TEM *A. awamori* (a and b) native cells, (c and d) Cu(II) – stressed cells.

Effect of Cu(II) on Peroxidase (POD), polyphenol oxidase (PPO) and glutathione reductase (GR) activities of A. awamori

The activities of POD, PPO and GR were measured to elucidate their role in coping with oxidative stress induced by Cu(II) exposure. Following the treatment with 50 and 100mg/l concentrations of Cu(II), complete inhibition in POD and PPO activities was observed (Fig. 5). Hossain & Kermasha (1998) reported that copper-metallothionein (Cu-MT) by donating copper, could act as an inhibitor for PPO activity in *A. niger*. Peroxidase and PPO activities increased with further increase in Cu(II) concentration stress and were found to be maximum at 300mg/l concentration of Cu(II) then reduced. An increase in POD and PPO activities indicateed that there was an effort on part of the fungus to minimize or neutralize the hazardous effect of ROS generated by Cu(II) treatment and that accumulation in the mycelium is required to induce the observed changes. The gradual decrease of enzyme activity suggests that the generated ROS caused irreversible oxidative damage in the fungal cell and inhibited POD activity (Guelfi *et al.*, 2003).

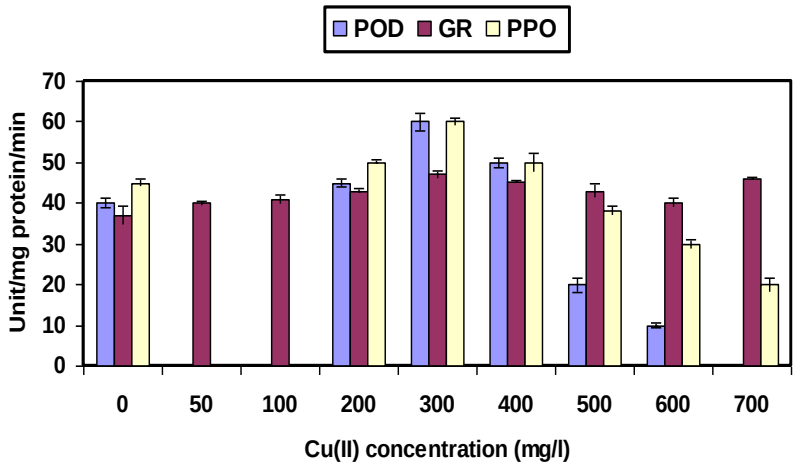

Fig. 5. Peroxidase (POD), polyphenol oxidase (PPO) and glutathiductase (GR) activity in *A. awamori* under different Cu(II) concentrations stress.

Glutathione, a tripeptide (-t-glutamyl-t- cystinylglycine), is one of the major antioxidant molecules of the cell, and is thought to play a vital role in buffering the cell against ROS (Stephen & Jamieson, 1996). Glutathione reductase (GR) also can remove H_2O_2 and maintains a balance between reduced glutathione (GSH) and oxidized glutathione (GSSG), as the latter is toxic to cells (Guelfi *et al.*, 2003). Activity of GR in *A. awamori* gradually increased by treatment with low concentrations of Cu(II) (Fig. 5). When the concentration of Cu(II) increased to 300mg/l, GR activity reached its maximum value of 47U/mg protein. Beyond this concentration, progressive decrease occurred. An increase in GR activity probably occurred to minimize the toxic effect of H_2O_2 and other ROS generated

due to Cu(II) toxicity. Comparable results were also obtained (Guelfi *et al.,* 2003; Ban *et al.,* 2012 and Chakraborty *et al.,* 2012).

Effect of Cu(II) on total antioxidant of A. awamori

Antioxidants (both enzymatic and nonenzymatic) provide protection against deleterious metal-mediated free radical attacks (Volka *et al.,* 2013). Thus, the influence of Cu(II) on non enzymatic antioxidant production was determined. After Cu(II) stress, a complete inhibition in nonenzymatic antioxidants was observed. In the case of Cu(II) stress, *A. awamori* was more tolerant as indicateed before. The toxic effect of Cu(II) may be diminished by efflux (Nies, 1999) or by the previously mentioned ultra structural changes like an increment in cell wall thickness and vacoulation. However it is apparent that enzymatic antioxidants had a greater role than nonenzymatic in alleviating stress induced by Cu(II).

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

Soluble protein exudation is assumed to be one of the mechanisms of heavy metal tolerance and varies depending on the species, the specific heavy metal and its concentration (Weishuang e*t al.,* 2009). Changes in the intracellular and extracellular soluble protein content in *A. awamori* under different concentrations of Cu(II) are shown in Fig.6. After Cu(II) treatment, extracellular protein content gradually increased while intracellular protein content decreased. The maximum values were observed at 400 mg /l concentration of Cu(II). *A. awamori* synthesized more protein up to certain level of Cu(II) treatment, a survival strategy which again may reflect the possibility of the Arndt-Schutrz 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 a more free flow of nutrients within cells and thus metabolic activity increases (Babich & Stotzky, 1980 and Ahonen- Jounarth *et al.,* 2004). 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.

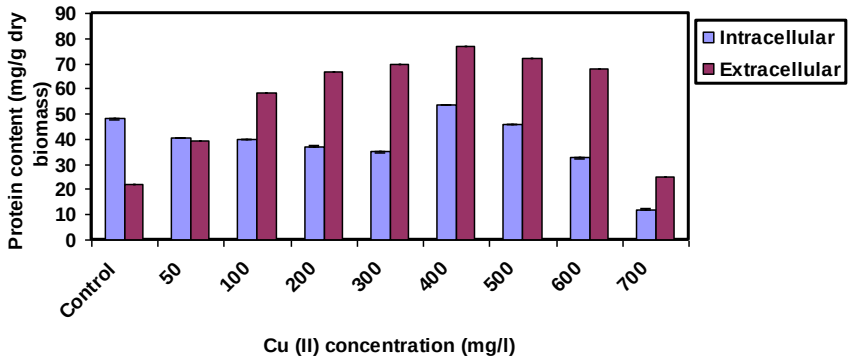

Fig. 6. Extracellular and intracellular protein content in *A. awamori* under different Cu(II) concentrations stress .

Effect of Cu(II) on thiol content of A. awamori

Thiol compounds play an important role in ROS perception. Immediate oxidation of protein SH-groups in the presence of ROS, diversity of the products produced (thiyl radical -S˙, disulfide -S-S-, sulfenic -SOH, sulfinic $-SO_2H$ and completely oxidized sulfonic acid $–SO_3H$ as well as formation of sulfenylamide or sulfonamide with the adjacent amino acid residues) and reversibility of these reactions make thiols the key compounds in reception and transduction of the ROS signal (Poole *et al.*, 2004). At 50mg/l concentration of Cu(II), there was an increase in extracellular thiol content (176.1% with respect to control) (Fig. 7). With further increase in Cu(II) concentration, a decrease in extracellular (but still higher than that of control) and intracellular thiol content was observed. Then, at 500-700mg/l concentrations of Cu(II), intracellular thiol content increased. An increase in thiol content indicateed the role of thiol in the tolerance or detoxification of Cu(II). The level of intracellular thiol content was found to be elevated during metal stress. Thiols are a well- known for metal chelation and detoxification. Some important members of the thiol family are capable of binding heavy metal ions to thiol- containing compounds such as GSH and sequestering these metal-thiol complexes into sub- cellular compartments of vacuoles (Schmoger *et al.*, 2000 and Ge *et al.*, 2011).

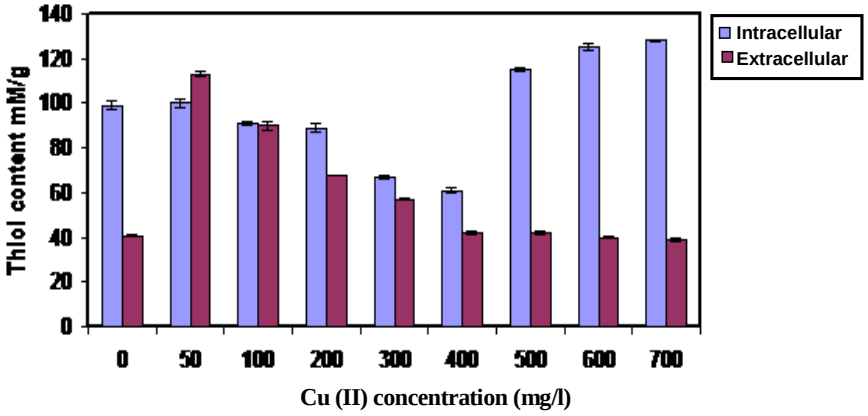

Fig. 7. Extracellular and intracellular thiol content in *A. awamori* under different Cu(II) concentration.

Oxalic acid secretion

Oxalate secretion is well-documented in both brown-rot, white-rot and *A.niger* and this process seems to be stimulated under Cu(II) stress (Clausen & Green, 2003 and Jarosz-Wilkolazka *et al.*, 2006). HPLC chromatograms of control and Cu(II)-stressed samples (Fig. 8a and b, respectively) revealed that the concentration of oxalic acid was found to be 16.1 and 28.9 mg/ml, respectively. These results indicate that Cu(II) stress stimulated the production of oxalic acid (79.5%, with respect to control). The production of oxalate in most brown-rot copper-tolerant fungi increased 2- to 17-fold when grown in wood preservative containing copper rather than in untreated wood (Green & Clausen, 2001). The

detoxification strategies of *A. foetidus* occurred by two mechanisms. One of them is the production of extracellular metabolites (*e.g.* citrate, oxalate) that is capable of adsorbing and precipitating the metal ions on the cell surface (Ge *et al.,* 2011).

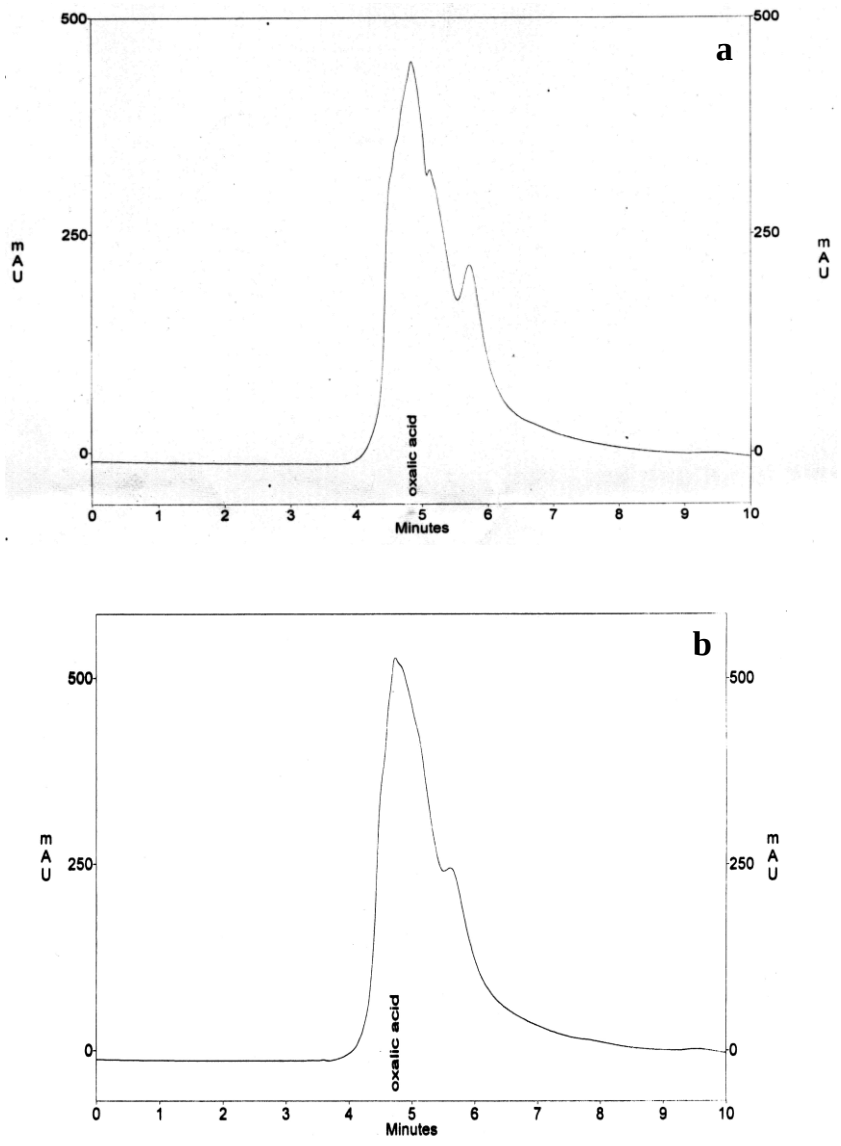

Fig. 8. HPLC chromatograms of *A. awamori* (a) native cells, (b) Cu(II) – stressed cells.

Biosorption results

Initial metal ion concentration

As the metal ions concentration increased, biosorption capacity also increased and reached saturation values of 73 and 50mg Cu(II) for live and dead biomass, respectively, at 500mg/l concentration of metal ions (Fig. 9). The uptake capacity was reduced with the rise in initial metal ion concentration to 700mg/l. At lower initial solute concentration, the ratio of the initial moles solute to the available surface area was low; subsequently, the fractional sorption become 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).

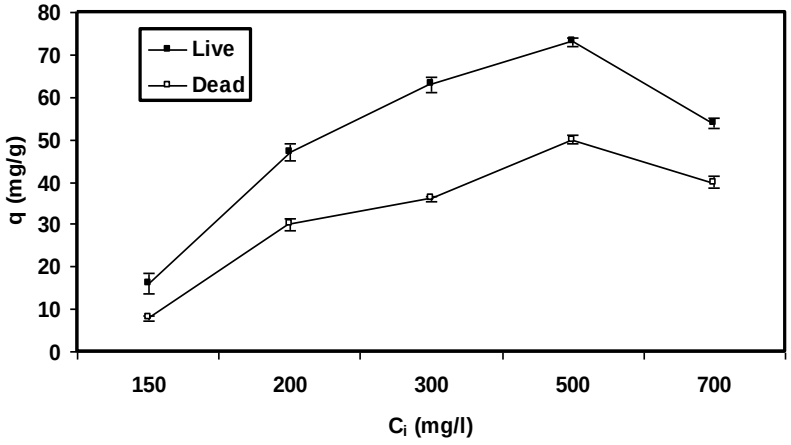

Fig. 9. Effect of initial metal ion concentration (C_i) on Cu(II) uptake capacity of live and dead biomass of *A. awamori*. Biosorption conditions: m = 1g/l; pH = 4.0; t= 30 min (for dead biomass) and 180 min (for live biomass).

Although *A. awamori* was tolerant for Cu(II), its biosorption capacity was not as high as expected. In this respect, Ruta *et al.* (2010) reported that the most of tolerant strains of yeast were hypo accumulators, owing to their gained tolerance to either reduced metal uptake or to enhanced export activity. Other explanation by Viraraghavan & Srinivasan (2011) was that the biosorption was higher for metals with larger ionic radius, the exceptions being chromium and the alkali metal ions. Ionic radius of Cu(II) = 128 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 solution pH of above 6.3, the Cu(II) ions precipitate and making the biosorption of Cu(II) impossible. As seen in Fig. 10, low pH (2.0) had more drastic effect on Cu(II) biosorption capacity of live cells (5.0 mg/g) than that of dead cells (13.0 mg/g). This is due to protein denaturation at low pH (Rothchild & Mancinelli, 2001). It can be seen that Cu(II) biosorption capacity increased with increasing initial pH and began to decline after reaching maximum value at pH 4.0 for dead and live biomass. A sudden increase in sorption with a slight increase in pH was observed and 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 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 4.0 might be attributed to the speciation of the metal ions, such as the formation of $Cu(OH)_2$ ions that do not adsorb well and resulted in the reduction of biosorption.

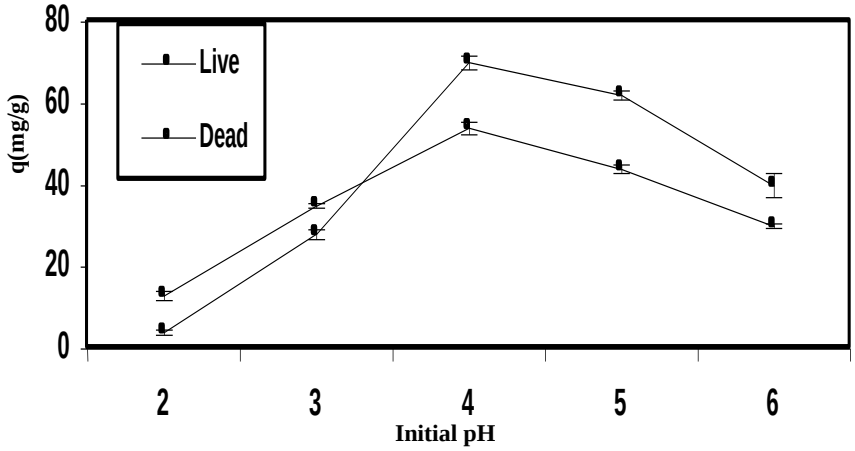

Fig. 10. Effect of initial pH on Cu(II) uptake capacity of live and dead biomass of *A. awamori*. Biosorption conditions: C_i = 500 mg/l; m = 1g/l; t = 30min (for dead biomass) and 180min (for live biomass).

Biosorbent concentration

The biosorption of Cu(II) with different biosorbent concentrations is shown in Fig. 11. The uptake capacities of live and dead biomass for Cu(II) decreased with increasing biomass concentration. The highest uptake capacities of live and dead biomass for Cu(II) were observed at 1g/l concentration. At a given equilibrium concentration, the biomass takes up more metal ions at lower versus higher cell densities (Mehta & Gaur, 2005). They suggested that electrostatic

interactions between cells can be a significant factor in the relationship between biomass concentration and metal sorption. It can be seen that the biosorption capacity decreased from 70 and 50 to 16 and 8mg/g for live and dead biomass, respectively, as the biomass concentration increased from 1 to 5 g/l. In this connection, Wang & 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.

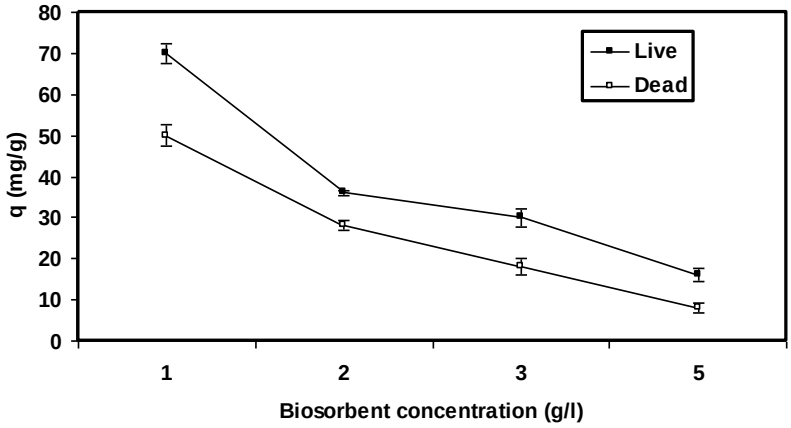

Fig. 11. Effect of biosorbent concentration (m) on Cu(II) uptake capacity of live and dead biomass of *A. awamori*. Biosorption conditions: C_i = 500 mg/l; pH = 4.0; t = 30min (for dead biomass) and 180min (for live biomass).

Contact time

The biosorption capacity of dead and live biomass as a function of contact time is presented in Fig. 12. It can be seen that the primary fast phase of biosorption by dead biomass occurred within the first 20 min and this was followed by second slow phase until equilibrium. A similar trend was observed (Wang *et al.*, 2010). Equilibrium time was found to be 30 min. On contrary, the rate of biosorption by live biomass was slow and reached the equilibrium within 180 min. Metal ion uptake by yeast is known to involve an initial rapid phase (passive uptake), followed by much slower phase (active uptake). 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 small part (Goyal *et al.*, 2003).

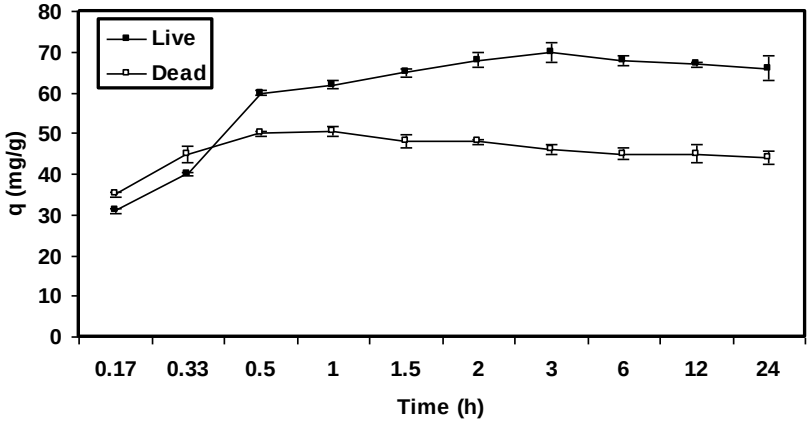

Fig. 12. Effect of contact time (t) on Cu(II) uptake capacity of live and dead biomass of *A. awamori*. Biosorption conditions: C_i = 500 mg/l; m = 1g/l and pH = 4.0.

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, sulhydryl, thioether, sulfonate, amine, imine, amide, imidazole, phosphonate and phosphodiester (Gupta & Mohaptra, 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 of native cells, Cu(II)-loaded live and Cu(II)-loaded dead cells in the range of 4000- 400cm^{-1} is shown in Fig. 13a-c. There are some guide peaks in the spectra of the control which are helpful for achieving this goal (Fig. 13a). The position or the intensities of these peaks are expected to be changed upon the interaction of biomass with Cu(II); also new peaks and shoulders are found in the spectra of Cu(II)-loaded samples which gave an indicateion on chelation between oxygen-, nitrogen-, sulphur-, or phosphorus containing ligands of biomass with metal ions. A marked shift occurred in the wave number from 3402 to 3392 and 3400 cm^{-1} in live and dead Cu(II)- loaded biomass (Fig. 13b and c, respectively). Such shifts in the wave number and intensities of these peaks indicatee the interaction of – NH_2 asymmetric stretch mode of amines and – OH groups with Cu(II) uptake. The changes in the intensities of peaks at 2926.5 cm^{-1} may be because of the interaction of –CH stretching vibrations of – CH_2- CH_2. Appearance of peak only in live Cu(II)-loaded biomass at wave number 2356.6 cm^{-1} indicatee the involvement of aliphatic chain –CH in Cu(II) uptake by live biomass. The disappearance of peak at 2857 cm^{-1} in case of live Cu(II)-loaded biomass and the shift in the intensity by 3% T in the case of dead Cu(II)-loaded biomass may be because of the interaction of – CH stretching vibrations of CH_2 – CH_3. After Cu(II) biosorption by live biomass, the peak at 1741.4cm^{-1} is replaced by shoulder at 1730 cm^{-1}, while in the case of dead biomass a

slight shift occurred. These changes indicateed the interaction of carbonyl stretch of unionized carboxylates. A small shift of bands at 1637 cm^{-1} with a significant increase in the intensity after metal ion uptake can be attributed to CO stretching mode conjugated to NH deformation mode and an amide I band. A slight shift of band at 1550 cm^{-1} with an increase in the intensity was observed. This was the result of amide II and NH deformation mode conjugated to C= N deformation mode. The marked shift at 1424 cm^{-1} for live Cu(II)-loaded biomass was indicateive of sulfonyl and sulfonamide groups. A small shift of bands at 1424 cm^{-1} was observed after Cu(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 1372 to 1377 cm^{-1} after Cu(II) uptake by live and dead biomass. FTIR spectra of live biosorbent incurred more changes than dead, indicateing that more functional groups were involved in the biosorption process. The disappearance of bands being located at 1153 and 1151.3 cm^{-1} which are attributed to the loading of Cu(II) was explained by the involvement of S=O stretching. Shifting of bands at 1081 cm^{-1} coupled with a sharp or significant decrease in the intensity and disappearance of bands at 1030.8cm^{-1} may be due to the interaction of adsorbed metal with sulfoxides, S=O stretching, sulfones, sulfonic acids and sulfonamides by live and dead biomass. Appearance of new bands after Cu(II) uptake by live and dead biomass at 865 and 860 cm^{-1}, respectively indicateed the intervention of phosphorus and P=S stretching in the process. The appearance of new bands at wave number ranging from 616.2 to 673.3cm^{-1} and a marked shift at 588.2 cm^{-1} after uptake of metal ions is indicateive of C-S stretching. The role of C-S stretching appeared again in either a marked shift or disappearance of band at 449.3cm^{-1} after metal ions uptake. Soft metals, such as Hg(II), Cd (II) and Pb(II) form stable bonds with nitrogen- and sulphur- containing (soft) ligands CN^-, R-S, SH^-, NH_2^- and imidazole (Wang & 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 biological ligands, generally in the order: S > N > 0 (Chen & Wang, 2007). Covalent index of Cu(II) = 2.98 (Brady & Tobin, 1995). Li *et al.* (2013) reported that the FTIR analysis of *Aspergillus* sp.J2 indicateed that the sulfur compound was involved in Se(IV) biosorption.

X- ray diffraction analysis(XRD)

To elucidate the chemical nature of *A. awamori* cell bound Cu(II), test biomass was subjected to XRD analysis before (control) and after Cu(II) sequestration (Fig. 14a-c). In contrast to untreated (control) biomass, which is expectedly amorphous (Fig. 14a), XRD spectra for live and dead biomass loaded with Cu(II) showed distinct reproducible patterns typical for the presence of crystalline materials (Fig 14b-c). Following Cd(II) and Cu(II) treatment, it was noticed that peaks in live biomass were more pronounced than these of dead one. The XRD pattern of Cu(II)-loaded live biomass (Fig. 14b) showed 28 peaks at 2θ ranging from 13.26 to 58.88A^o and corresponding to respective d-spacing from 6.68 to 1.57A^o. The XRD pattern of Cu(II)-loaded dead biomass (Fig. 14c) showed 23 peaks at 2θ ranging from 18.23 to 59.08 A^o and corresponding to respective d-spacing from 4.87 to 1.56 A^o. Based on spacing d-values, these peaks after Cu(II) uptake are attributed to the presence of crystalline Cu(II) and Cd(II) sulphate hydrate; $CuSO_4.H_2O$. FTIR spectroscopic analysis of the tested

biomass also confirmed the involvement of sulfur-oxygen compounds in these metal ions uptake.

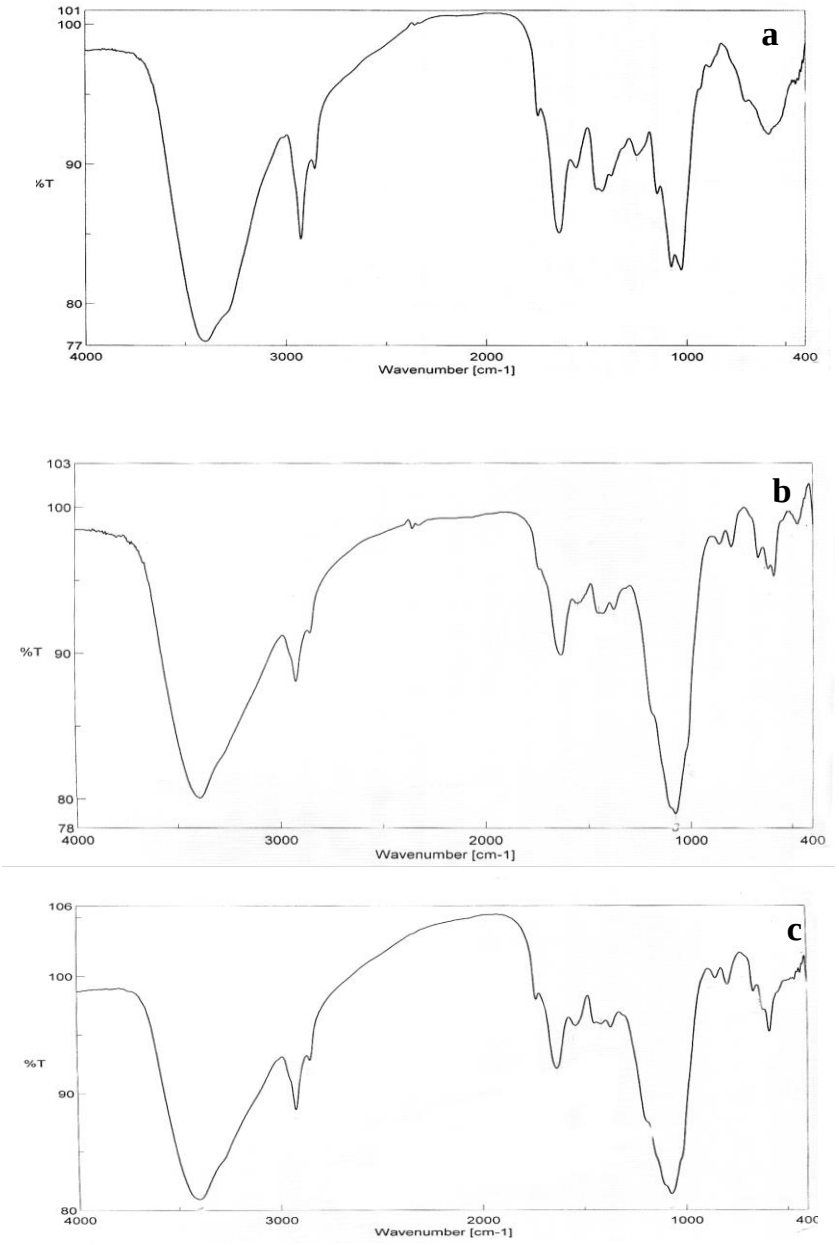

Fig. 13. FTIR spectra of *A. awamori.* (a) Native cells, (b) Cu(II) – loaded live cells, (c) Cu(II) – loaded dead cells.

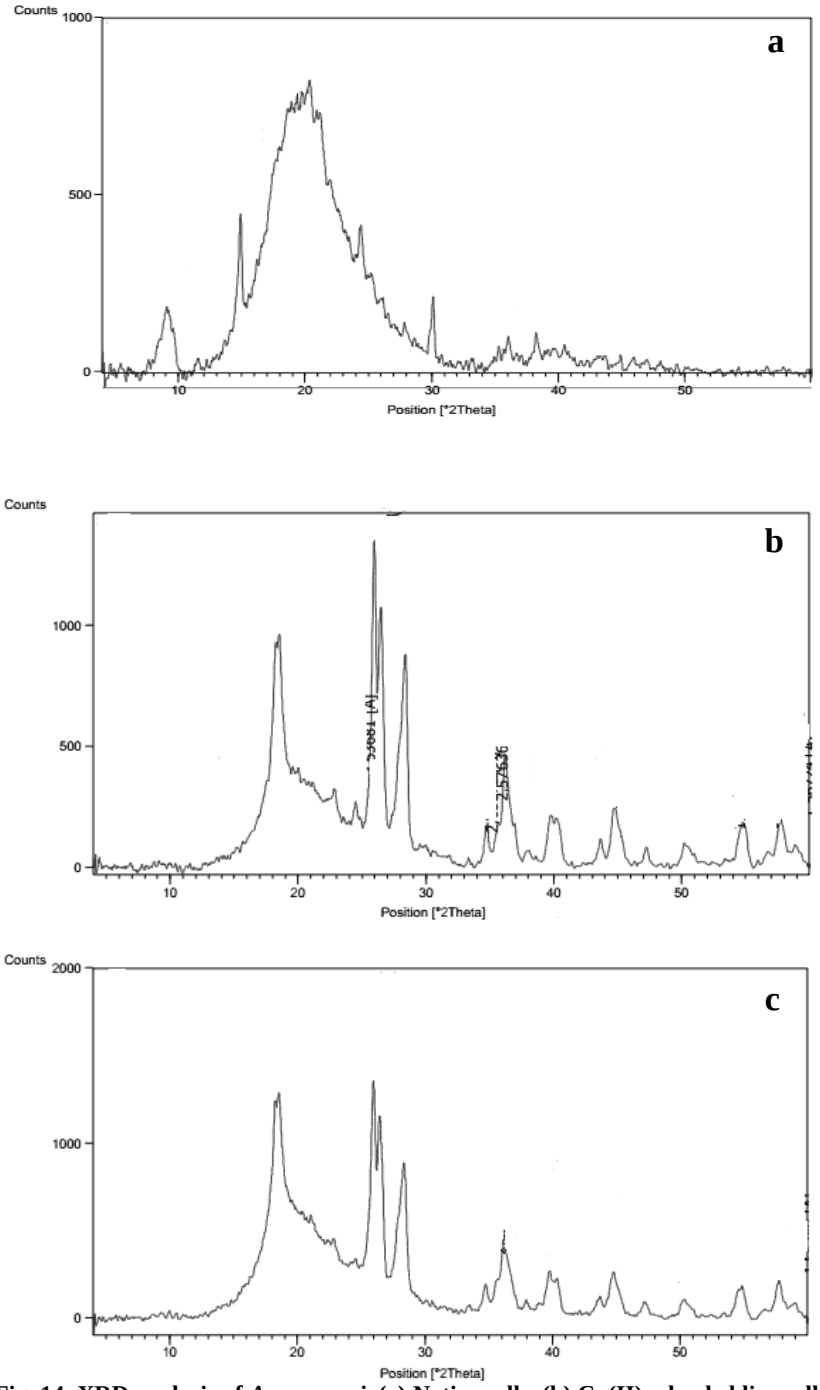

Fig. 14. XRD analysis of *A. awamori.* (a) Native cells, (b) Cu(II) – loaded live cells, (c) Cu(II) – loaded dead cells.

Energy dispersive-X-ray microanalysis (EDAX)

EDAX 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 various angles that can then be co-related with the complex formed (Gupta & Mohapatra, 2003). X-ray peaks showed a broadening in the peak full wave height maximum (FWHM) which confirmed the size of pellets of $CuSO_4$. H_2O compounds. Also the height of the peak should be proportional to the weight percent of the compounds or their ingredients; Cu(II), S and O. This bulk technique gives an elemental ratio of the population as a whole when analyzing the whole pellet following the metal exposure. EDAX spectra of Cu(II)-loaded live and dead biomass showed distinct peaks for oxygen, sulfur and Cu(II) with element % 43.7, 6.1, 50.2, 67.02, 6.99 and 25.99, respectively. EDAX spectra of Cu(II)-loaded live and dead biomass (Fig. 15a and b) showed distinct peaks for oxygen, sulfur and Cu(II) with element % 53.56, 11.36, 35.09, 72.29, 15.23 and 12.48, respectively. EDAX showed an excellent agreement with the corresponding FTIR and XRD analyses. FTIR indicateed the involvement of cellular sulfur-oxygen compounds in both metal ions binding. XRD analysis confirmed the presence of $CuSO_4.H_2O$ on live and dead biomass.

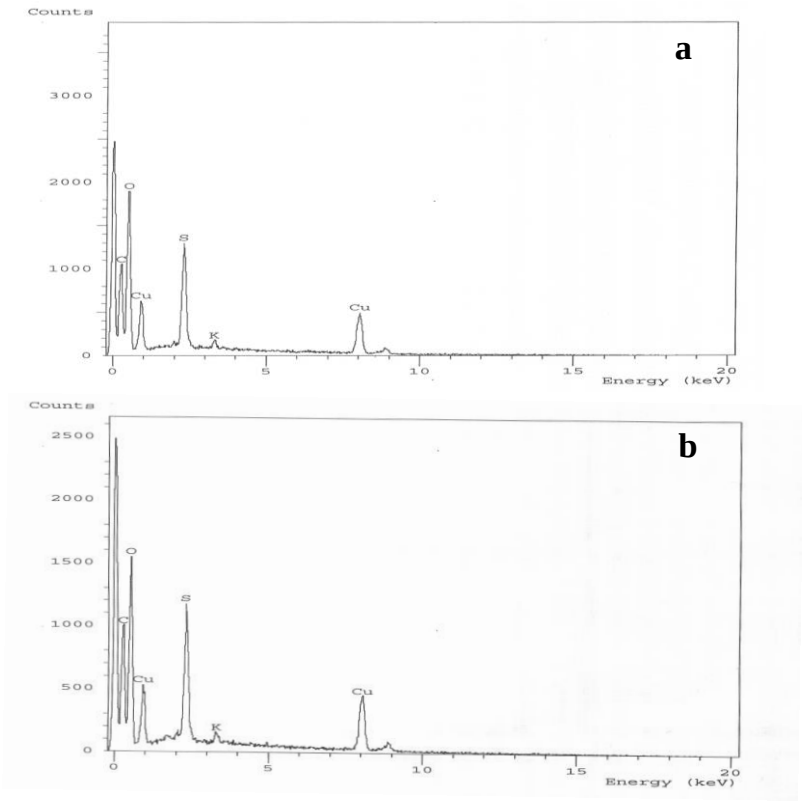

Fig. 15. EDAX microanalysis of *A. awamori.* (a) Cu(II) – loaded live cells, (b) Cu(II) – loaded dead cells.

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(*Received* 17/9/2013;
accepted 18/11/2013)

دراسه على التحمل والقدره على الإمتصاص لفطرة *اسبيرجلس اوماری* لعنصر النحاس

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تم تعيين درجة التثبيط الصغرى وكانت 3000 ملليجرام/لتر وحدث تثبيط كامل لإنتاج البيوماس على الوسط الغذائى السائل عند 800 ملليجرام/لتر.بواسطة الفحص بإستخدام الميكرسكوب الإلكترونى الماسح لوحظت تغييرات للفطره المعامله بعنصر النحاس.تم اثبات وجود النحاس على البيوماس بإستخدام اديكس.الفحص بإستخدام الميكرسكوب الإلكترونى النافذ اثبت حدوث الإدمصاص الخارج خلوى والنفاذيه لداخل الخلايا وتكوين فجوات.تم دراسة التغيرات فى نشاط انزيمات البولى فينول اوكسيديز وجلوتاثيون ريدكتيز والبيرواوكسيديز وفى تركيزات البروتين الكلى ومضادات الأكسده الكليه نتيجه لوجود تركيزات مختلفه من عنصر النحاس.تم دراسة اثر العوامل المختلفه على قدرة الفطر على الإمتصاص.استخدمت تقنية FTIR,XRD,EDAX لمعرفة آلية الإمتصاص لعنصر النحاس.