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Multiple metal tolerance and biosorption of cadmium by *Candida tropicalis* isolated from industrial effluents: glutathione as detoxifying agent

Abdul Rehman · Muhammad Sohail Anjum

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Abstract The ability of cadmium uptake by metal-resistant yeast, *Candida tropicalis*, from the liquid medium and wastewater was evaluated. The minimum inhibitory concentration of Cd^{2+} against *C. tropicalis* was 2,500 mg L^{-1}. The yeast also showed tolerance toward Zn^{2+} (1,400 mg L^{-1}), Ni^{2+} (1,000 mg L^{-1}), Hg^{2+} (1,400 mg L^{-1}), Cu^{2+} (1,000 mg L^{-1}), Cr^{6+} (1,200 mg L^{-1}), and Pb^{2+} (1,000 mg L^{-1}). The yeast isolate showed typical growth curves, but lag and log phases extended in the presence of cadmium. The yeast isolate showed optimum growth at 30°C and pH 8. The metal processing ability of the isolate was determined in a medium containing 100 mg L^{-1} of Cd^{2+}. *C. tropicalis* could decline Cd^{2+} 70%, 85%, and 92% from the medium after 48, 96, and 144 h, respectively. *C. tropicalis* was also able to remove Cd^{2+} 40% and 78% from the wastewater after 6 and 12 days, respectively. Cd produced an increase in glutathione (GSH) and nonprotein thiol levels by 135% and 134% at 100-mg L^{-1} concentration, respectively. An increase in the synthesis of GSH is involved in metal tolerance, and the presence of increasing GSH concentrations may be a marker for high metal stress in *C. tropicalis*. *C. tropicalis*, which is resistant to heavy metal ions and is adaptable to the local environmental conditions, may be employed for metal detoxification operations.

Keywords Cadmium · Heavy metal · Glutathione · Metabolic inhibitors · *Candida tropicalis* · Bioremediation

A. Rehman (✉) · M. S. Anjum
Department of Microbiology and Molecular Genetics,
University of the Punjab, New Campus,
Lahore 54590, Pakistan
e-mail: rehman@mmg.pu.edu.pk

Introduction

Heavy metals are the most abundant pollutants in the sewage and in wastewater (Hong et al. 1996) and are one of the main causes of water and soil pollution (Nriagu and Pacyna 1988). Anthropogenic use of metals has resulted in the extensive contamination of freshwater ecosystems that has had harmful effects on fauna. Human activities, such as mining operations and the discharge of industrial wastes, have resulted in the accumulation of metals in the environment and eventually are accumulated through the food chain, leading to serious ecological and health problems (Cheng 2003).

Cadmium (Cd) is a heavy metal contaminant in the environment. It is extensively used in the industry for a number of applications, including electroplating, protection against corrosion, and stabilizing plastic and batteries (Lebrun et al. 1994). It is also obtained as a by-product of zinc

production (Nies 1999). Cd has been utilized eight times more during the last 40 years by mankind than in its entire history; the Cd^{2+} input into the biosphere is estimated to be about 30,000 tons/year (Nriagu and Pacyna 1988).

Cadmium ions are nonbiodegradable and tend to accumulate in living organisms, causing various disorders (Nogaw and Kido 1996). Cd is carcinogenic, embryotoxic, teratogenic, and mutagenic and may cause hyperglycemia, reduced immunopotency, and anemia, due to its interference with iron metabolism (Sanders 1986). The toxicity of Cd has also been well documented in selective types of almost all major phyla of eukaryotes (Unger and Roesijadi 1996; Coeurdassier et al. 2004).

The conventional wastewater treatment technologies, such as electrochemical treatment, ion exchange, oxidation–reduction, and membrane separation, are very expensive and have several disadvantages, such as unpredictable metal ion removal, high reagent requirements, and generation of toxic sludge, which are often difficult to dewater and require extreme caution at the time of there disposing (Siloniz et al. 2002). Bioremediation, which involves the use of microbes to detoxify and degrade environmental contaminants, has received increasing attention in recent times to clean up a polluted environment (Malik 2004; Rehman et al. 2008). Bioremediation provides a safe and economic alternative to commonly used physiochemical strategies (Eccles 1995). The use of microbial biomass of bacteria (Shakoori and Qureshi 2000), fungi (Rehman et al. 2008), and algae (Feng and Aldrich 2004) for removal of heavy metals from aqueous solution is gaining attention. Microorganisms with the ability to grow in the presence of heavy metals and with a significant metal uptake have a potential use in bioremediation of polluted waters (Shakoori et al. 2004).

Glutathione (GSH) is widespread in bacteria, plants, and animals (Meister and Anderson 1983) and participates in some significant cellular activities including the protection of cells against toxic metals. GSH in *Saccharomyces cerevisiae* may account for 1% of the cell dry weight (Penninckx and Elskens 1993), where it functions as a storage form of endogenous sulfur and nitrogen (Mehdi and Penninckx 1997) as well as having a role in protection against biocides and certain metal ions (Rama-Rao et al. 1997). The postulated defensive action of GSH against metal toxicity was primarily based on observed cellular accumulation in response to metal ions.

Active uptake systems can take up both essential and nonessential metal ions and thus are of interest in bio-removal. The essential characteristics of a living biomass used in a metal ion removal process are tolerance and uptake capacities (Macaskie and Dean 1989; Suh et al. 1998). Yeast biomass is an inexpensive, readily available source of biomass. Furthermore, yeast cells retain their ability to accumulate a broad range of heavy metals to varying degrees under a wide range of external conditions (Villegas et al. 2005).

The objectives of this study were to isolate cadmium-resistant yeast from polluted areas, investigate cadmium stress on yeast growth and resistance to other metal ions, measure glutathione and nonprotein thiol levels, and ascertain the ability of yeast isolate to remove cadmium from the culture medium and wastewater.

Materials and methods

Sample collection

Wastewater samples were collected in screw-capped sterilized bottles from industrial area of Sheikhupura, a small town located 40 km central west of Lahore, Pakistan, known for its industry. The industrial effluents are discharged in open land, rendering the atmosphere absolutely smelly and the air unbreathable. Some physicochemical parameters of wastewater, viz., temperature (degrees Celsius), pH, and dissolved cadmium (milligrams per liter) were measured (APHA 1989).

Isolation of cadmium-resistant yeast

For isolation of cadmium-tolerant yeasts, 100 μL of the wastewater sample was spread on yeast extract, peptone, and dextrose (YEPD) agar plates containing 0.05-mg L^{-1} Cd^{2+} of the medium. YEPD agar plates were prepared by dissolving 1 g of yeast extract, 0.5-g peptone, and 0.2-g glucose in 100-mL distilled water, pH adjusted

at 7.2 to 7.5, and then, 1.5-g agar was added in the 250-mL flasks. The medium was autoclaved at 121°C and 15-lb (6.8 kg) pressure for 15 min. The growth of the yeast colonies was observed after 48 h of incubation at 30°C. Isolated colonies were picked up with sterilized wire loop and streaked on YEPD agar medium plate containing 100-mg Cd^{2+} L^{-1}. It was again incubated at 30°C for 48 h. This process was repeated with successively higher concentrations of Cd^{2+} (150-, 200-, and 250- up to 2,500-mg Cd^{2+} L^{-1}) until the minimum inhibitory concentration (MIC) of each isolate was obtained. The MIC is defined as the lowest concentration of Cd^{2+} at which a single colony-derived streak could not grow.

Physical, biochemical, and molecular characterizations of the yeast isolate

The yeast isolate was tested for colony morphology, spore staining, starch hydrolysis, ester production, nitrate reduction, yeast–malt agar test, citrate utilization, acid production from glucose, ammonia from urea, fermentation of carbohydrates, and tolerance of 1% acetic acid. For physical and biochemical characterizations of yeast isolate, the criteria adopted by Benson (1989) were followed. For further identification, genomic DNA was isolated (Masneuf-Pomarèdge et al. 2007), and the 18S ribosomal RNA (rRNA) gene was amplified by polymerase chain reaction (PCR) using two general yeast 18S rRNA primers (ITS-5; 5′-GGAAGTAAAAGTCGTAACAACG-3′, ITS-4; 5′-TCCTCCGCTTATTGATATGC-3′). PCR reaction conditions were as per Larena et al. (1999). The PCR product (approx. 0.58 kb) was cleaned up using a Fermentas purification kit (#K0513). Sequencing was carried out by CEQ-800 Genetic Analysis System (Beckman), Coulter Inc., Fullerton, CA, USA. Nucleotide sequence similarities were determined using the basic local alignment search tool (BLAST; National Center for Biotechnology Information database; http://www.ncbi.nlm.nih.gov/BLAST). The sequence was aligned with close matches using ExPasy-Tools (Clustal. W) and multiple sequence alignment program (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and a dendrogram based on the homologous sequences was created using the same package.

Determination of optimum pH and temperature

For optimum growth of the yeast isolate, two parameters, i.e., temperature and pH, were considered. For determination of optimum temperature, a 5-ml YEPD broth was added in five sets, each of three test tubes, autoclaved and inoculated with 20 μL of freshly prepared culture of yeast isolate. The five sets of tubes were incubated at 20°C, 25°C, 30°C, 35°C, and 40°C. After an incubation of 16 h, the absorbance was taken at 600 nm using a LAMBDA 650 UV/Vis Spectrophotometer (PerkinElmer, USA).

For determination of optimum pH, test tubes having a 5-ml YEPD broth were prepared in seven sets, each containing three test tubes, and pH was adjusted at 4, 5, 6, 7, 8, 9, and 10 then autoclaved. These tubes were inoculated with 20-μL freshly prepared culture of the yeast isolate. After an incubation period of 16 h, the absorbance was taken at 600 nm.

Growth curves of yeast isolate

Effect of Cd^{2+} on the growth of yeast isolate was determined in YEPD medium supplemented with Cd^{2+} (100 mg L^{-1}). For yeast isolate, a 100-mL YEPD broth was taken in two sets consisting of three flasks, autoclaved, and then one set (three flasks, control) inoculated with 100 μL of the freshly prepared inoculum, but no metal ions were added in the medium. The other three flasks (treated), inoculated with 100 μL of inoculum, were maintained at a concentration of 100-mg Cd^{2+} L^{-1}. These flasks were incubated at 30°C in a shaker at 60–80 rpm. An aliquot of culture was taken out in an oven sterilized tube, at regular intervals of 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, and 48 h. Absorbance was taken at 600 nm.

Cross metal resistance

The cross heavy metal resistance of yeast isolate was performed to evaluate the ability of yeast isolate to tolerate other metal ions. For this purpose, stock solution of 10 g L^{-1} of different

metal salts (cadmium chloride, copper sulfate, lead nitrate, nickel chloride, mercuric chloride, potassium dichromate, and zinc sulfate) was used. Cd was present at concentration of 100 mg L^{-1} in solutions that were used to check the minimum inhibitory concentration against respective metals. Metals were added separately in the medium. The cross metal resistance was determined by increasing the concentration of respective metal in a stepwise manner with 100 mg L^{-1} of metal-checked resistance. This procedure was repeated with higher concentration of each metal ion in the medium. Every time, 100 mg L^{-1} of each metal ion was added more than that of the previous step, until the MIC of each metal was obtained. Inoculated cultures, containing metal ions, were incubated at 30°C for a maximum period of four days. Experiment was repeated in triplicate.

Estimation of Cd^{2+} processing ability of yeast isolate

The metal processing capability of yeast isolate was checked by adding Cd^{2+} at a concentration of 100 mg L^{-1} in the defined culture medium ([grams per liter]: d-glucose 30, yeast autolysate 7.5, peptone 7.5, NH_4Cl 9, $KH_2{\cdot}PO_4$ 2.75, $MgCl_2{\cdot}2H_2O$ 2, $FeSO_4{\cdot}7H_2O$ 0.002, and K_2HPO_4 5.2 [pH 7.2-7.5]; Kujan et al. 2005) to minimize the complexation of the heavy metal ions. The control culture medium was also run for Cd containing the same concentration as in the treated one, i.e., 100 mg L^{-1} but was without the yeast isolate. The cultures were incubated at 30°C, and from each medium (control and treated), 5-mL culture was taken out under sterilized conditions after 48, 96, and 144 h, respectively. The cultures were spun down at 3,000 rpm for 5 min, and the supernatants were used for the estimation of Cd by atomic absorption spectrophotometer (Varian, USA) at a wavelength of 228.8 nm. In the present study, metal uptake values were determined from the difference in final metal concentration between the control flask without cells and the test flask with cells at different time periods. The amount of metal in the supernatants was determined by using standard curve. The percentage decrease in the amount of Cd in the medium was calculated.

Removal of cadmium from industrial effluents

To check the efficacy of yeast cells to remove Cd from semisynthetic wastewater, a lab-scale experiment was set up. Three plastic containers were taken. In the first container, 10 L of tap water was taken along with 1.5 L of yeast isolate grown to log phase. In the second container, 10 L of industrial effluent was taken along with 1.5 L of 48-h grown yeast culture. In the third container, only 10 L of industrial effluent was taken, and 100 mg L^{-1} of Cd stress was maintained in each container. Experiment was carried out at room temperature (25 ± 2°C). After 6 and 12 days of incubation, three samples from each container were taken, centrifugated to separate the cells, and supernatants used to estimate the amount of Cd in wastewater and the quantity removed by the yeast cells.

Uptake of cadmium in the presence of metabolic inhibitors

The experiment was performed to check the effect of inhibitors on the metal uptake ability of the yeast isolate. Cd removal was monitored in the presence of 2,4-dinitrophenol (DNP, 1 mM) and *N*, *N*′-dicyclohexylcarbodiimide (DCC, 100 μM; Li et al. 2008). Yeast cells were grown in 500-mL Erlenmeyer flasks containing 100 mL of YEPD medium with 1% inoculum. Flasks were incubated on an orbital shaker at 30°C and agitated at 150 rpm. At the midexponential growth phase, inhibitor was added to the liquid medium. Both inhibitors were added 15 min before the addition of Cd (100 mg L^{-1}).

Samples (10 mL) were withdrawn from the culture flasks at defined intervals harvested by centrifugation at 6,000 rpm (EBA 20, Hettich Zentrifugen) for 10 min, and the supernatants were transferred to sterilized test tubes and were used for metal estimation. The pellet was washed three times with distilled water, and the pellet obtained was divided into two portions. One portion was washed three times in 0.1-M ethylenediaminetetraacetic acid (EDTA) for 10 min. The amount of Cd associated with the cell biomass was removed as the EDTA washable fraction presents at the cell surface, thereby allowing

only intracellular Cd to be measured. The second portion was washed with distilled water and centrifuged at 14,000 rpm for 5 min. After centrifugation, supernatant was discarded. The pellet was further used for acid digestion by adding 0.2-N HNO_3 (1:1; 200 μL) and left it overnight for complete acid digestion. Acid-digested tubes were used for estimation of total metal content, i.e., both the metal adsorbed on the surface of the organism as well as that taken intracellularly. Intracellular Cd was determined by subtracting the amount of absorbed metal from the total metal content.

Estimation of glutathione and other nonprotein thiol contents

Reduced glutathione (GSH), oxidized glutathione (GSSG), and total glutathione contents were determined according to Israr et al. (2006). Briefly, 100 mL of YEPD broth medium in each of three 250-mL flasks, two treated and one as control, was inoculated with 1 mL of fresh yeast culture and incubated at 30°C. After 24 h, Cd was added to 100-mg L^{-1} treated flask and incubated at 30°C. After 48 h of growth, the cells were pelleted at 6,000 rpm (EBA 20, Hettich Zentrifugen) and weighed on a weighing balance (UX 320G, Shimadzu Corporation, Japan). One milliliter of 5% sulfosalicyclic acid was added, and the cells were sonicated (Heilscher Ultrasonic Processors UP 400, S) at 4°C two to three times for 15 s with 60-s interval. The sonicate was centrifuged at 14,000 rpm for 10 min, and supernatant was used for estimation of glutathione and nonprotein thiols.

For estimation of GSH, 0.5 mL of reaction buffer (0.1-M phosphate buffer [pH 7], 0.5-mM EDTA) was added in 0.5 mL of above aliquot, and 50 μL of 3-mM 5′-dithio-bis-(2-nitrobenzoic acid) was added. After 5 min, absorbance was taken at 412 nm. In the same Eppendorf tube, 100 μL of 0.4 mM of NADPH and 2-μL glutathione reductase (GR) were added. After 20 min, optical density was taken at 412 nm for the determination of total glutathione. The amount of GSSG was calculated by subtracting GSH from total glutathione concentrations. A standard curve was prepared from varying concentrations of reduced glutathione. One hundred microliters of the aliquot was placed in Eppendorf tubes, and 0.5 mL of reaction buffer (0.1-M phosphate buffer [pH 7], 0.5-mM EDTA) and 0.5 mL of 1-mM 5′-dithio-bis-(2-nitrobenzoic acid) were added. The reaction mixture was incubated for 10 min, and optical density was taken at 412 nm. Values were corrected for the absorbance by preparing a blank without extract. A standard curve was prepared from varying concentrations of cysteine to calculate the other nonprotein thiol contents in samples.

Statistical analysis

Observations were made, and all the experiments were repeated two or more times. The results reported are average values. Standard deviation and standard error of the mean were also calculated.

Results and discussion

Physicochemical characteristics of industrial wastewater

Some physicochemical characteristics of industrial wastewater were ascertained, from where metal-tolerant yeast was isolated. The temperature of different samples ranged between 25°C and 39°C, pH ranged between 6.0 and 9.5, dissolved oxygen between 0.287 ± 0.01 and 1.56 ± 0.01 mg L^{-1}, Cd^{2+} ranged between 0.0186 ± 0.03 and 0.0241 ± 0.01 mg L^{-1}, Cu^{2+} ranged between 0.0126 ± 0.01 and 0.0201 ± 0.01 mg L^{-1}, Hg^{2+} ranged between 0.0106 ± 0.01 and 0.0074 ± 0.03 mg L^{-1}, Ni^{2+} ranged between 0.0112 ± 0.03 and 0.0067 ± 0.03 mg L^{-1}, and Cr^{6+} ranged between 0.0254 ± 0.01 and 0.0135 ± 0.03 mg L^{-1}. Extensive industrialization has brought about huge changes in the distribution of elements at the surface of the Earth. Metal-resistant microorganisms become dominant in habitats contaminated with relevant heavy metals.

Identification of yeast isolate

The morphological and biochemical characteristics of the yeast isolate are shown in Table 1. The partially amplified (580 bp) and sequenced

Table 1 Morphological and biochemical characteristics of the yeast isolate

Characters	Yeast isolate
Shape	Round
Size	0.1–0.35 mm
Color	Creamy off white
Texture	Shiny
Margin	Round
Elevation	Raised
Type	Budding
Starch hydrolysis	–
Ester production	+
Citrate utilization	–
Tolerance of 1% acetic acid	–
Acid production from glucose	–
Production of ammonia from urea	–
Nitrate reduction	+
Sugar fermentation (glucose, sucrose, and maltose)	+
	+
	+

+ positive, – negative

18S rRNA gene from the local isolate was uploaded to the National Center for Biotechnology Information website to search for similarity to known DNA sequences and to confirm the species of the locally isolated yeast. The BLAST query revealed that this gene is 100% homologous to the already reported gene of *C. tropicalis* strain American Type Culture Collection 750 (AY939810). The nucleotide sequences coding for the 18S rRNA gene of *C. tropicalis* have been submitted to the GenBank database under accession number EU 924133. Other close matches included *C. tropicalis* voucher MCCC2E00325, EF196807 (gi/124126988; 99% similarity), *C. tropicalis* EU288196 (gi/162424886; 99% similarity), *C. tropicalis* EF216862 (gi/125381275; 99% similarity), and *C. tropicalis* DQ680841 (gi/110564235; 98% similarity). Dendrogram indicates (Fig. 1) the identity of the isolate as a species of *Candida*.

Optimum pH and temperature

The most suitable temperature for the growth of the Cd-resistant yeast isolate was found to be 30°C. Maximum growth for *C. tropicalis* was observed at pH 8. The growth curve pattern was studied by growing the *C. tropicalis* in the presence of Cd^{2+} (100 mg L^{-1}) and comparing with the control culture in which no metal ions were added. The growth pattern of *C. tropicalis* (control) was significantly different from the growth pattern of the yeast isolate in the presence of Cd^{2+}. It is interesting to note that the lag phase of the yeast isolate was extended from 4 to 8 h in Cd-treated culture medium. The growth pattern is shown in Fig. 2.

In this study, growth rate of yeast isolate in the presence of Cd^{2+} was lower as compared with that of nontreated yeast culture. Slow growth of the yeast *Candida intermedia* strain was observed at 50-mg L^{-1} copper, and the lag growth phase was very extended (Fujs et al. 2005). Specific growth rates in yeasts were decreased at different concentrations of cadmium, copper, and zinc but depending on the type of yeast (Balsalobre et al. 2003).

Heavy metal resistance

C. tropicalis was found to be resistant to Cd up to a concentration of 2,500 mg L^{-1}. The yeast could also tolerate Cu^{2+} (1,000 mg L^{-1}), Cr^{6+} (1,200 mg L^{-1}), Hg^{2+} (1,400 mg L^{-1}), Ni^{2+} (1,000 mg L^{-1}), Pb^{2+} (1,000 mg L^{-1}), and Zn^{2+} (1,400 mg L^{-1}). The order of resistance regarding the metal concentration was $Cd^{2+} > Zn^{2+} = Hg^{2+} > Cr^{6+} > Cu^{2+} = Ni^{2+} = Pb^{2+}$.

Zafar et al. (2007) reported the metal tolerance and biosorption potential of filamentous fungi isolated from metal-contaminated agricultural soil, and tolerance among filamentous fungi was observed in order of Cu > Cr > Cd > Co > Ni. Li and Yuan (2006) reported that *Rhodotorula* sp. Y11 was resistant and highest metal uptake value obtained was 19.38 mg g^{-1} by boiling treated yeast cells. *C. albicans* and *C. tropicalis* are known for high levels of resistance to the water-soluble ions Hg^{2+}, Pb^{2+}, Cd^{2+}, arsenate (AsO_4^{3-}), and selenite (SeO_3^{2-}; Berdicevsky et al. 1993). Moreover, the tolerance to metals and the capacity of the uptake are dependent on ionic metal and yeast species (Balsalobre et al. 2003).

Metal processing ability

Cd processing capability of the yeast isolate was checked by adding Cd^{2+} at 100 mg L^{-1} in the

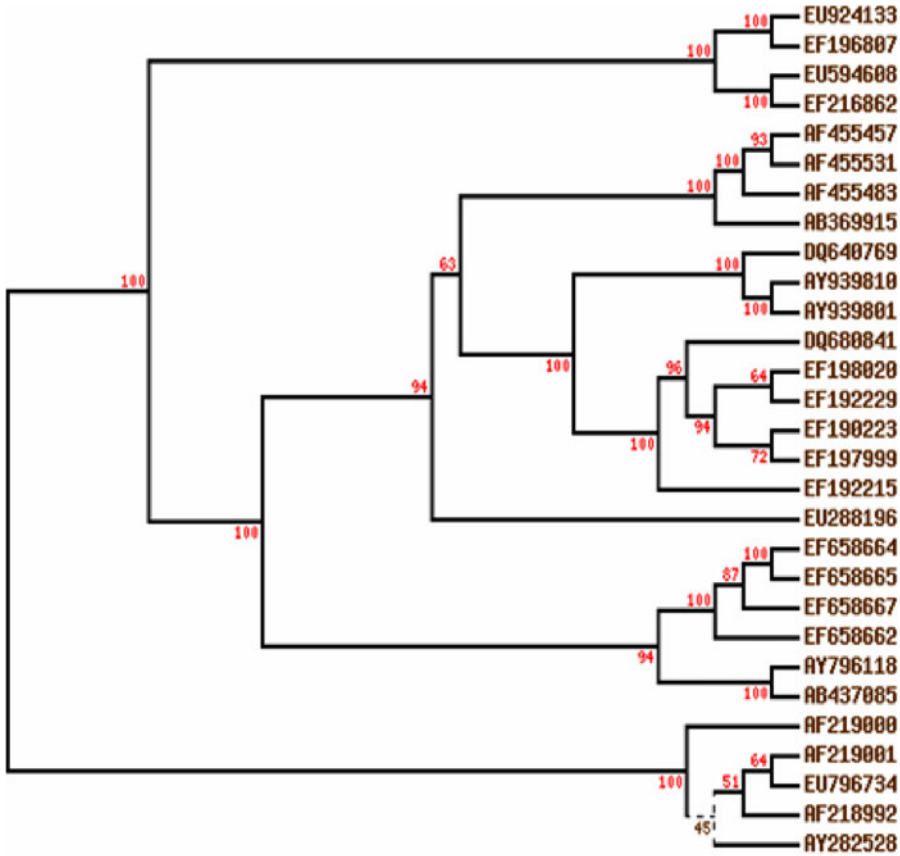

Fig. 1 Dendrogram showing the similarity of *C. tropicalis* with other close members of *Candida* sp.

culture medium. *C. tropicalis* could remove 92% of Cd from the medium after 144 h of incubation. The yeast was also capable to decrease Cd^{2+} by 70% and 85% from the medium after 48 and 96 h, respectively (Fig. 3).

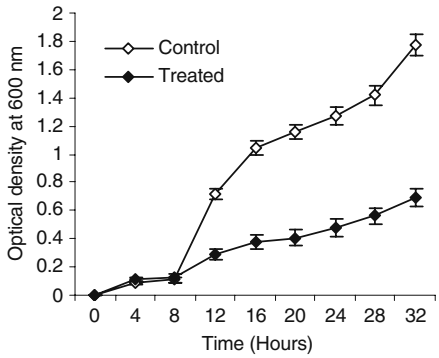

Fig. 2 Effect of Cd^{2+} concentration (100 mg L^{-1}) on the cell growth of *C. tropicalis* in YEPD medium after incubation at 30°C

Cd tolerance and bioaccumulation have been studied in fungi (Holan and Volesky 1995; Yan and Viraraghavan 2003; Li and Yuan 2006). Rehman et al. (2007) reported that *C. tropicalis* culture grown in the medium containing Cu^{2+} (100 mg L^{-1}) could decline to 74% of copper from the medium after 96 h of incubation. The yeast was also capable of decreasing Cu^{2+} by 16%, 20%, 29%, 43%, 46%, 55%, and 68% from the medium after 6, 12, 18, 24, 30, 48 and 72 h, respectively. Many *Candida* spp. also have the capacity to adsorb and/or accumulate metals from their surroundings.

Removal of Cd from industrial effluents

To assess the ability of the yeast *C. tropicalis* to remove Cd^{2+} from contaminated industrial effluents, a large-scale experiment was performed. Industrial wastewaters harbor a variety of microorganisms including bacteria, fungi, algae, and

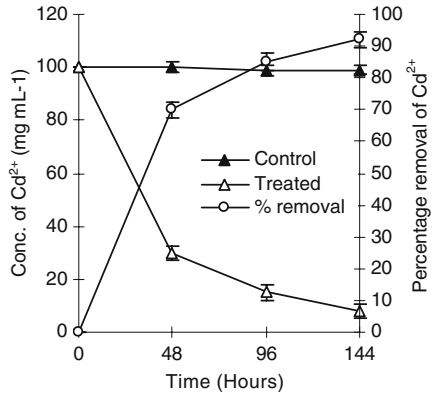

Fig. 3 Biosorption of Cd by *C. tropicalis* in YEPD medium incubated at 30°C. The yeast cells were inoculated in three 250-mL flasks with 100 mL of defined culture medium containing 100 μg mL^{-1} of cadmium. The control did not contain cells of the yeast. Five-milliliter culture was taken out from each flask 48, 96, and 144 h after inoculation and spun down, and the supernatant was used for cadmium estimation with an atomic absorption spectrophotometer. Mean of three readings and percentage removal from the medium were plotted against time in hours

ciliates. *C. tropicalis* was observed to remove 40% Cd from the wastewater after 6 days and was also able to remove 78% from the wastewater after 12 days. The yeast took up 28 and 60 mg L^{-1} of Cd from distilled water containing 100 mg L^{-1} Cd within 6 and 12 days, respectively (Fig. 4). Rehman et al. (2007) reported that *C. tropicalis* was observed to remove 64% copper from the

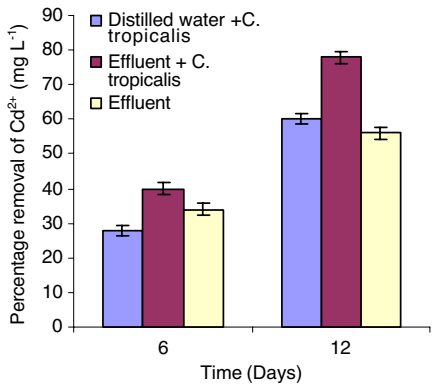

Fig. 4 Percentage removal of Cd^{2+} by *C. tropicalis* from 10-L aqueous solutions (distilled water and industrial effluent) with initial concentration of 100 mg L^{-1} of Cd^{2+} after 6 and 12 days of incubation at room temperature

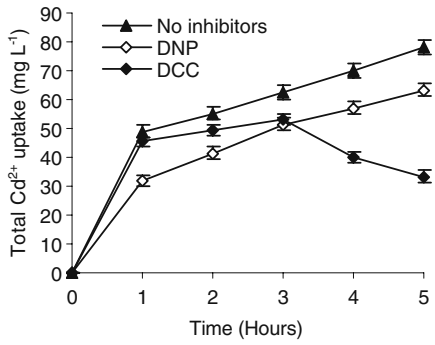

Fig. 5 Total uptake of Cd by *C. tropicalis* in the presence of metabolic inhibitors

industrial wastewater after 4 days and 74% after 8 days.

Cadmium uptake in the presence of metabolic inhibitors

The total cadmium uptake was fairly decreased in the presence of metabolic inhibitors during 5 h of the accumulation process. Total cadmium uptake was 78.05 mg L^{-1} in the control medium, while in DNP and DCC, the Cd uptakes were 63.35 and 39.9 mg L^{-1}, respectively (Fig. 5). The decline in total Cd uptake in the presence of DNP and DCC was 19% and 49%, respectively. Figure 6 shows the intracellular Cd concentration in yeast biomass with and without metabolic inhibitors. The maximum Cd contents measured in yeast cells containing no inhibitors were 50.55 mg L^{-1}. Maximum Cd uptakes from the medium

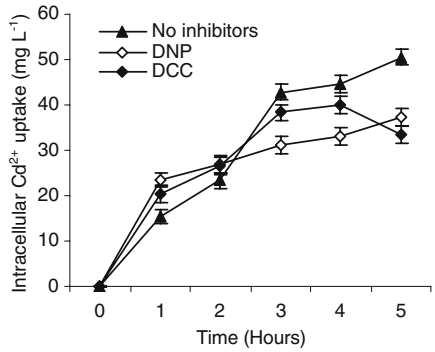

Fig. 6 Intracellular uptake of Cd by *C. tropicalis* in the presence of metabolic inhibitors

containing DNP and DCC were 37.3 and 33.4 mg L^{-1}, respectively.

The intracellular Cd accumulation was also greatly inhibited by the presence of inhibitors. The percent declines in intracellular Cd accumulation both in DNP and DCC were 26% and 34%, respectively. This low concentration of Cd accumulation in the presence of inhibitors indicates that Cd accumulation requires adenosine triphosphatase (ATPase) activity in *C. tropicalis*. Accumulation of Cd in yeast cells was significantly reduced in the presence of both metabolic inhibitors DNP and DCC, indicating its dependence on ATPase activity. Similar results were also reported by other workers (Malekzadeh et al. 2002; Li et al. 2008). Yeast growth in the presence of metabolic inhibitors indicates metabolism-dependent and metabolism-independent processes of the heavy metal resistance mechanisms.

Measurement of GSH and non-protein thiols

Cd treatment altered the levels of GSH and GSSG in *C. tropicalis*. Cd at a concentration of 100 mg L^{-1} significantly increased GSH level (135%) and GSH/GSSG ratio (3.5) with respect to the control. The level of nonprotein thiols also showed a significant increase (134%) in Cd-treated yeast cells (Table 2). Cd produced an increase in GSH and nonprotein thiol levels by 135% and 134% at 100 mg L^{-1} concentration, respectively.

Microbial detoxification of metal ions is achieved by several mechanisms including regulation of uptake, transformation into less toxic species, and intracellular immobilization (Rama-Rao et al. 1997; Gharieb and Gadd 1998). GSH is present in most living cells and has been shown to play numerous roles, such as protection against oxidative stress, detoxification, transport, and enzymatic catalysis (Penninckx 2002; Kim et al. 2005). In yeast, the main defense mechanism against Cd consists of exporting the GSH–metal complex into the vacuole; this compartmentation requires energy and involves the protein Ycf1 (Li et al. 1997). However, the destination of this complex inside the vacuole is unknown.

The present investigation clearly demonstrates the importance of glutathione and non-protein thiols in Cd accumulation and detoxification. Intracellular concentration of GSSG increases at the expense of GSH under stress conditions. Cd treatment significantly enhanced the GSH/GSSG ratio at 100 mg L^{-1} of Cd. This indicates the potential of *C. tropicalis* to tolerate Cd stress. Cd treatment also increased the contents of nonprotein thiols in *C. tropicalis*, and this increase in nonprotein thiols was almost the same as that in GSH. Thiols are essential agents in cellular redox signaling and control in animals, plants, and fungi (Moran et al. 2001; Pócsi et al. 2004). Delhaize et al. (1989) reported that glutathione decreased Cd^{2+} toxicity and was also present at low levels in a Cd-tolerant strain of *Datura innoxia* compared with a sensitive strain. The intracellular glutathione in mice functions in protection against Cd^{2+} toxicity, and this tripeptide provides the first line of defense against Cd before induction of metallothionein synthesis occurs (Singhal et al. 1987). Gharieb and Gadd (2004) reported the direct evidence for the involvement of glutathione in Cd detoxification in *S. cerevisiae*. A relationship between tolerance and reduced cellular content of Cd was shown: the mutant strain accumulated approximately two-fold more Cd than that accumulated by the wild-type strain. This suggests an influence of GSH on cellular uptake of Cd and also directly confirms the protective action of such a cellular thiol

Table 2 Levels of reduced (GSH) and oxidized glutathione (GSSG), total glutathione, reduced and oxidized glutathione ratio, and nonprotein thiols in *C. tropicalis* exposed to Cd at 100 mg L^{-1}

Cd Concentration (mg L^{-1})	GSH (mM g^{-1}FW)	GSSG (mM g^{-1}FW)	GSH+GSSG (mM g^{-1}FW)	GSH/GSSG ratio	Nonprotein thiols	Percent increase in nonprotein thiols
0	18.75 ± 0.6[a]	15.75 ± 1.2	34.50 ± 1.2	1.20 ± 0.8	3.075 ± 0.6	7.20–3.075: 4.125 = 134%
100	63.00 ± 0.8	18.00 ± 0.5	81.00 ± 1.6	3.50 ± 0.6	7.20 ± 0.8	

[a] ±SE ($n = 3$)

compound against Cd toxicity. Smith et al. (2007) reported that cell-to-cell variation in the stress resistance of *S. cerevisiae*, particularly to cadmium, was revealed to depend on the antioxidant glutathione.

In the present investigation, *C. tropicalis* showed high resistance against different heavy metal ions ranging from 1,000 to 2,500 mg L^{-1}. *C. tropicalis* accumulated substantial amount of Cd^{2+} from the medium (92%) and wastewater (78%) and is adaptable to the local environmental conditions. Therefore, *C. tropicalis* may be applicable for the treatment of cadmium-containing wastewater.

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