Full Paper

Cadmium biosorption by yeast, *Candida tropicalis* CBL-1, isolated from industrial wastewater

Abdul Rehman,* Muhammad Sohail Anjum, and Shahida Hasnain

Department of Microbiology and Molecular Genetics, University of the Punjab, New Campus, Lahore 54590, Pakistan

(Received December 25, 2009; Accepted April 30, 2010)

The present study is aimed at assessing the ability of metal-resistant yeast, *Candida tropicalis* CBL-1, to uptake metal from liquid medium. The minimum inhibitory concentration of Cd(II) against *Candida tropicalis* CBL-1 was 2,800 mg/L. The yeast could also tolerate Zn(II) (3,100 mg/L), Hg(II) (2,400 mg/L), Ni(II) (2,200 mg/L), Cr(VI) (2,000 mg/L), Pb(II) (1,100 mg/L), and Cu(II) (2,200 mg/L). 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 7. The metal processing ability of the isolate was determined in a medium containing 100 mg/L of Cd(II). *Candida tropicalis* CBL-1, could reduce Cd(II) 59%, 64% and 70% from the medium after 48, 96 and 144 h, respectively. *C. tropicalis* CBL-1 was also able to remove Cd(II) 46% and 60% from the wastewater after 6 and 12 days, respectively. Cd produced an increase in glutathione and non-protein thiols level by 37% (17.50 ± 0.8 – 24.0 ± 1.2) and 18% (3.30 ± 0.7 – 3.90 ± 0.8) at 100 mg/L concentration, respectively. Metal tolerance and accumulation together with changes in the GSH status and non-protein thiols under Cd exposure were studied in *C. tropicalis*.

Key Words—bioremediation; cadmium; *Candida tropicalis* CBL-1; glutathione; metabolic inhibitors

Introduction

Heavy metal release to the environment has been increasing continuously as a result of industrial activities and technological development and poses a significant threat to the environment, public and soil health (Cerbasi and Yetis, 2001). Industrial effluents are generated from hundreds of small and large lock manufacturing and plating industries and contain considerable amounts of heavy metals at elevated concentrations (Malik and Jaiswal, 2000).

Cadmium (Cd) is known to be a non-essential element, and can be toxic at very low concentrations.

This metal is also ubiquitous in sewage sludges, industrial wastes and mining sites. Cd is highly toxic because of its strong affinity to purines, pyrimidines, phosphates, porphyrins, and the cysteine and histidine residues of proteins. The mechanism of Cd toxicity includes binding to –SH groups, competing with Zn and Se for inclusion into metalloenzymes and competing with calcium for binding sites (calmodulin). In kidneys, free Cd binds to kidney glomerulus and results in proximal tubule dysfunction. Cadmium causes lung toxicity. It is responsible for edema and emphysema by killing lung macrophages. Its effects on skeletal muscles include osteoporosis and osteomalacia. About 8% of lung cancers are caused by Cd (Jin et al., 2003; Klaassen, 2002).

Traditional technologies 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

* Address reprint requests to: Dr. Abdul Rehman, Department of Microbiology and Molecular Genetics, University of the Punjab, New Campus, Lahore 54590, Pakistan.

Tel: 92-42-9231249

E-mail: rehman@mmg.pu.edu.pk; rehman_mmg@yahoo.com

dewater and require extreme caution when disposing of them (Siloniz et al., 2002a). The use of microbial biomass of bacteria (Shakoori and Qureshi, 2000), fungi (Rehman et al., 2007a) and algae (Feng and Aldrich, 2004) for removal of heavy metals from aqueous solution is gaining increasing attention. The high surface to volume ratio of microorganisms and their ability to detoxify metals are among the reasons that they are considered as a potential alternative to synthetic resins for remediation of dilute solutions of metals and solid wastes (Kapoor et al., 1999).

Active uptake systems can take up both essential and non-essential 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 (Suh et al., 1998). One of the most ubiquitous biomass types available for bioremediation of heavy metals at low pH is yeast. 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 aim of this work was to isolate cadmium resistant yeasts from polluted areas, investigate the responses of growing yeast to cadmium stress and to ascertain the ability of yeast isolates to remove cadmium from a culture medium and wastewater.

Materials and Methods

Sample collection. Wastewater samples were collected in screw capped sterilized bottles from an industrial area of Sheikhpura (Pakistan). Some physicochemical parameters of wastewater viz., temperature ($^{\circ}\text{C}$), pH and cadmium (mg/L) were measured (APHA, 1992).

Isolation of cadmium-resistant yeast. For isolation of cadmium tolerant yeasts, 100 μL of the wastewater sample was spread on YEPD (yeast extract, peptone and dextrose) agar plates containing 0.05 mg of Cd(II)/L 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, adjusting the pH to 7.2 to 7.5 and then adding 1.5 g agar to 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 loops and streaked on YEPD agar medium plates containing 100 mg Cd(II)/L . They were again incubated at 30°C for 48 h. This process was repeated with successively higher concentrations of Cd(II) (150, 200, and 250 up to 2,800 mg Cd(II)/L) until the minimum inhibitory concentration (MIC) of each isolate was obtained. The MIC is defined as the lowest concentration of Cd(II) at which a single colony-derived streak could not grow.

Physical, biochemical and molecular characterization of the yeast isolate. For biochemical characterization the yeast isolate was tested for colony morphology, spore staining, starch hydrolysis, ester production, nitrate reduction, citrate utilization, acid production from glucose, ammonia from urea, fermentation of carbohydrates, and tolerance of 1% acetic acid. For physical and biochemical characterization of yeast isolate the criteria adopted by Benson (1994) were followed. For further identification, genomic DNA was isolated and the 18S rRNA gene was amplified by PCR using two general yeast 18S rRNA primers (ITS-5; 5'-GGAAGTAAAAGTCGTAACAACG-3', ITS-4; 5'-TCC TCCGCTTATTGATATGC-3'). PCR reaction conditions were as per Larena et al. (1999). The PCR product of (approx. 0.58 kb) was cleaned up using a Fermentas purification kit (#K0513). Sequencing was carried out by Genetic analysis system model CEQ-800 (Beckman), Coulter, Inc., Fullerton, CA, USA. Nucleotide sequence similarities were determined using BLAST (NCBI database; <http://www.ncbi.nlm.nih.gov/BLAST>). The sequence was aligned with close matches using the ExPasy-Tools (Clustal W) multiple sequence alignment program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

Determination of optimum growth conditions. For optimum growth of the yeast isolate, two parameters, i.e. temperature and pH, were considered. For determination of optimum temperature, 5 ml YEPD broth was added to 5 sets of three test tubes each, 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 5 ml YEPD broth were prepared in 7 sets, each containing 3 test tubes, and pH was adjusted at 4, 5, 6, 7, 8, 9 or 10 and then autoclaved. These tubes were inoculated with 20 μL freshly pre-

pared culture of the yeast isolate. After an incubation period of 16 h, the absorbance was taken at 600 nm.

Growth curves of yeast isolate. The effect of Cd(II) on the growth of yeast isolate was determined in YEPD medium supplemented with Cd(II) (100 mg/L). For yeast isolate 100 ml YEPD broth was taken in two sets consisting of 3 flasks, autoclaved and then one set (3 flasks, control) inoculated with 100 μ l of the freshly prepared inoculum but no metal ions were added to the medium. The other three flasks (treated), inoculated with 100 μ l of inoculum, were maintained at a concentration of 100 mg Cd(II)/L. 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 the yeast isolate was determined by using stock solutions of 10 g/L of different metal salts (cadmium chloride, copper sulphate, lead nitrate, nickel chloride, mercuric chloride, potassium dichromate and zinc sulphate). Cadmium was present at concentration of 100 mg/L in solutions that were used to check the minimum inhibitory concentration against respective metals. Metals were added separately to the medium. The cross metal resistance was determined by increasing the concentration of the respective metal in a stepwise manner with 100 mg/L of metal checked resistance. This procedure was repeated with higher concentrations of each metal ion in the medium. Every time, 100 mg/L more of each metal ion was added than in 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 4 days.

Estimation of Cd(II) processing ability of yeast isolate. The metal processing capability of the yeast isolate was checked by adding Cd(II) at a concentration of 100 mg/L in the defined culture medium [(g/L): D-glucose 30; yeast autolysate 7.5; peptone 7.5; NH_4Cl 9; KH_2PO_4 2.75; $\text{MgCl}_2 \cdot 2\text{H}_2\text{O}$ 2; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002; 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 cadmium containing the same concentration as the treated one, i.e. 100 mg/L but was without the yeast isolate. The cultures were incubated at 30°C for 96 h and from each medium (control and treated) 5 ml culture was taken out under sterilized conditions after 48, 96 and 144 h.

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 a test flask with cells at different time periods. The amount of metal in the supernatants was determined by using a standard curve. The percentage reduction in the amount of Cd in the medium was calculated.

Removal of cadmium on a large scale. To check the efficacy of yeast cells to remove cadmium from 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 (temperature, 32.5°C; pH, 7.4; dissolved oxygen, 0.00146 ± 0.04 g/L; Cu(II), 1.370 ± 0.03 mg/L; and Cd(II), 1.34 ± 0.03 mg/L) 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 of Cd stress was maintained in each container. The experiment was carried out at room temperature ($25 \pm 2^\circ\text{C}$). After 6 and 12 days of incubation samples were taken, centrifuged to separate the cells, and the 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. This experiment was performed to check the effect of inhibitors on the metal uptake ability of the yeast isolate. Cadmium removal was monitored in the presence of 2,4-dinitrophenol (DNP, 1 mM) and *N,N'*-dicyclohexylcarbodiimide (DCC, 100 μ M). 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 mid-exponential growth phase, inhibitor was added to the liquid medium. Both inhibitors were added 15 min before the addition of cadmium (100 mg/L).

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 EDTA for 10 min. The amount of cadmium associated with the cell biomass was removed as the EDTA washable fraction present 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, the supernatant was discarded. The pellet was further used for acid digestion by adding 0.2 N HNO₃ (1 : 1) (almost 200 µl), and left overnight for complete acid digestion. Acid digested tubes were used for estimation of total metal content, i.e., 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 non-protein thiol content. Reduced glutathione (GSH), oxidized glutathione (GSSG) and the total glutathione contents were determined according to Israr et al. (2006) with some modification. 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, Cd²⁺ was added at 100 mg/L to the treated flasks and incubated at 30°C. After 48 h, the cultures were centrifuged at $1,275 \times g$ (EBA 20, Hettich Zentrifugen) and weighed (UX 320G, Shimadzu Corporation, Japan). One milliliter of 5% sulfosalicylic acid was added and the cells were sonicated (Heilscher Ultrasonic Processors UP 400, S) at 4°C 2–3 times for 15 s with a 60 s interval. The sonicate was centrifuged at $1,120 \times g$ for 10 min. The supernatant was used for estimation of glutathione and non-protein thiols.

For estimation of GSH, 0.5 ml of reaction buffer [0.1 M phosphate buffer (pH 7), 0.5 mM EDTA] was added to 0.5 ml of the 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 NADPH₂ and 2 µl glutathione reductase (GR) was added. After 20 min, O.D 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. A standard curve was prepared from varying concentrations of cysteine to calculate other non-protein thiol content in samples.

Statistical analysis. Observations were made and all the experiments were repeated two or more times; the results reported are average values. The notable significant differences were calculated using Student's *t*-test wherever applicable.

Results

Physicochemical characteristics of industrial wastewater

Some physicochemical characteristics of industrial wastewater were ascertained, from where the metal tolerant yeast was isolated. The temperature of different samples ranged between 28°C and 39°C, pH ranged between 5.5 and 9.0, dissolved oxygen between 0.340 ± 0.01 and 1.35 ± 0.01 mg/L and Cd²⁺ between 1.43 ± 0.04 and 1.82 ± 0.01 mg/L.

Screening of Cd-resistant yeast

The wastewater samples were spread on YEPD agar plates supplemented with 100 mg/L of cadmium. Morphologically six different colonies were observed. Metal stress was increased in a stepwise manner and finally two isolates capable of growth at high cadmium concentration were selected and designated as CBLy-Cd1 and CBLy-Cd2. CBLy-Cd1 and CBLy-Cd2 were found to resist cadmium at concentrations of 2,800 and 1,800 mg/L, respectively. The highest Cd-resistant yeast isolate (CBLy-Cd1) was used for further study.

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 18S rRNA gene from the local isolate (CBLy-Cd1) was uploaded to the NCBI (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 an already reported gene of *Candida tropicalis* strain ATCC 750 (AY 939810.1). The nucleotide sequences coding for the 18S rRNA gene of *C. tropicalis* CBL-1 have been submitted to the GenBank database under accession number EU 594607. Other close matches included *C. tropicalis* isolate UR 9344-03, AY939801 (gi/65306744; 100% similarity), *C. tropicalis* EU288196 (gi/162424886; 100% similarity), *C. tropicalis* strain WC65-1 EF190223 (gi/124365216; 100% similarity), *C. tropicalis* strain w-14-1 EF198020

Table 1. Morphological and biochemical characteristics of yeast isolate.

Characters	<i>Candida tropicalis</i>
Shape	Round
Size	0.1–0.24 mm
Color	Off-white
Texture	Dull
Margin	Convex
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, maltose)	+

(+) positive; (–) negative.

(gi/122059244; 99% similarity) and *C. tropicalis* EF216862 (gi/125381275; 98% similarity).

Optimum growth conditions

The most suitable temperature for the growth of the cadmium-resistant yeast isolate was found to be 30°C. Maximum growth for *C. tropicalis* CBL-1 was observed at pH 7. The growth curve pattern was studied by growing *C. tropicalis* CBL-1 in the presence of Cd(II) (100 mg/L) and comparing it with the control culture to which no metal ions were added. The growth pattern of *C. tropicalis* CBL-1 (control) was significantly different from the growth pattern of the yeast isolate in the presence of Cd(II). It is interesting to note that the lag phase of the yeast isolate was extended in Cd-treated culture medium. The growth pattern is shown in Fig. 1.

Heavy metal resistance

C. tropicalis CBL-1 was found to be resistant to cadmium up to a concentration of 2,800 mg/L. The yeast could also tolerate Cu(II) (2,200 mg/L), Cr(VI) (2,000 mg/L), Hg(II) (2,400 mg/L), Ni(II) (2,200 mg/L), Pb(II) (1,100 mg/L) and Zn(II) (3,100 mg/L). *C. tropicalis* CBL-1 showed maximum resistance against Zn(II) at a concentration of 3,100 mg/L and the order of resistance regarding the metal concentration (mM) was

Fig. 1. Effect of Cd(II) concentration (100 mg/L) on the cell growth of *C. tropicalis* CBL-1 in YEPD medium after incubation at 30°C.

Fig. 2. Biosorption of Cd by *C. tropicalis* CBL-1 in defined culture 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 of cadmium. The control did not contain cells of the yeast. A five ml culture was taken out from each flask 48, 96 and 144 h after inoculation, spun down and the supernatant used for cadmium estimation with an Atomic Absorption Spectrophotometer. The mean of three readings and % age removal from the medium were plotted against time in hours.

Cu(II) > Zn(II) > Ni(II) > Hg(II) > Cr(VI) > Pb(II).

Metal processing ability

The cadmium processing capability the yeast isolate was checked by adding Cd(II) at 100 mg/L to the culture medium (Fig. 2). *C. tropicalis* CBL-1 could remove 70% of cadmium from the medium after 144 h of incubation. The yeast was also capable of decreasing Cd(II) ions by 59%, and 64% from the medium after 48 and 96 h, respectively.

Removal of cadmium from aqueous solutions at lab-scale

In order to assess the ability of the yeast *C. tropicalis* CBL-1 to remove Cd(II) ions from contaminated indus-

trial effluents a lab-scale experiment was performed. *C. tropicalis* CBL-1 was observed to remove 46% of cadmium from the wastewater after 6 days and was also able to remove 60% from the wastewater after 12 days. The yeast took up 29 and 42 mg/L of cadmium from distilled water containing 100 mg Cd/L within 6 and 12 days, respectively (Fig. 3). The original flora present in the wastewater was able to remove 20 and 30 mg/L Cd(II) after 6 and 12 days, respectively which was 56 and 50% less than the removal of Cd(II) from the original wastewater containing *C. tropicalis* (Fig. 3). This clearly indicates that *C. tropicalis* not only survived in the industrial effluents but also efficiently removed Cd(II) from the wastewater.

Cadmium uptake in the presence of metabolic inhibitors

The total cadmium uptake was appreciably reduced in the presence of metabolic inhibitors during the 5 h of accumulation process. Total cadmium uptake was 83 mg/L in the control medium while in DNP and DCC the Cd uptake was 59.1 and 68.7 mg/L, respectively

(Fig. 4). The reduction in total Cd uptake in the presence of DNP and DCC was 28.9% and 17.23%, respectively. Figure 4 also shows the intracellular cadmium concentration in yeast biomass with and without metabolic inhibitors. The maximum Cd contents (52.5 mg/L) were measured in the medium containing no inhibitors. Maximum Cd uptake from the medium containing DNP and DCC was 20.5 and 35.2 mg/L, respectively.

Measurement of GSH and non-protein thiols

Cd treatment altered the levels of GSH and GSSG in *C. tropicalis* CBL-1 (Table 2). Cd at a concentration of 100 mg/L significantly increased the GSH level (37%) and GSH/GSSG ratio (117.65%) with respect to the control. The NPSH content in the presence of 100 mg Cd/L was 3.90 ± 0.8 $\mu\text{mol/g}$ compared with the control 3.30 ± 0.7 $\mu\text{mol/g}$. The level of NPSH also showed a significant increase (18%) in Cd-treated yeast cells. Cd produced an increase in GSH and non-protein thiol levels by 37 and 18% at the 100 mg/L concentration, respectively.

Fig. 3. Percentage removal of Cd(II) by *C. tropicalis* CBL-1 from 10 L aqueous solutions (distilled water and industrial effluent) with initial concentration of 100 mg/L of Cd(II) after 6 and 12 days of incubation at room temperature.

Fig. 4. Total and intracellular uptake of cadmium by *C. tropicalis* CBL-1 in the presence of metabolic inhibitors.

Table 2. Levels of reduced (GSH) and oxidized glutathione (GSSG), total glutathione, reduced and oxidized glutathione ratio and non-protein thiols in yeast isolate exposed to Cd at 100 mg/L.

Cd conc. (mg/L)	GSH (mM g ⁻¹ FW)	GSSG (mM g ⁻¹ FW)	GSH+GSSG (mM g ⁻¹ FW)	GSH/GSSG ratio
0	17.50±0.8*	10.25±0.6	27.75±1.6	1.70±0.4
100	24.0±1.2	16.50±0.5	40.50±1.8	3.70±0.6

*mean $\pm$ SE (n=3).

Discussion

In the present study the growth rate of the yeast isolate in the presence of Cd(II) was lower as compared with that of a non-treated yeast culture. This happened because of a higher concentration of metals that probably poisoned essential biochemical reactions (Perego and Howell, 1997). The growth period was delayed when the concentration of heavy metal was increased (Brady et al., 1994). Slow growth of the yeast *Candida intermedia* strain was observed at 50 mg/L 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, depending on the type of yeast (Balsalobre et al., 2003).

Microbial detoxification of metal ions is achieved by several mechanisms including regulation of uptake, transformation into less toxic species and intracellular immobilization (Gharieb and Gadd, 1998; Rama Rao et al., 1997). Glutathione (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 (Kim et al., 2005; Penninckx, 2002). 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 study clearly indicates 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 of Cd. This indicates the potential of *C. tropicalis* CBL-1 to tolerate Cd stress. Cd treatment also increased the contents of NPSH in *C. tropicalis* CBL-1 and this increase in NPSH was less than that in GSH as a result of Cd treatment. At a concentration of 100 mg/L of Cd, the contents of NPSH increased by 18%, while GSH increased by 37%, compared to the control. An increase in GSH and NPSH under Cd stress indicates the role of thiols and GSH in Cd tolerance and accumulation in *C. tropicalis* CBL-1. Thiols are essential agents in cellular redox signaling and control in animals, plants and fungi (Moran et al., 2001; Noctor et al., 2002; Pócsi et al., 2004). Delhaize et al. (1989) reported that glutathione decreased Cd(II)

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(II) 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 direct evidence for the involvement of glutathione in Cd detoxification in *Saccharomyces cerevisiae*.

Removal of heavy metals from wastewater is normally achieved by advanced technologies such as ion exchange, chemical precipitation, ultra filtration, or electrochemical deposition. These technologies do not seem to be economically feasible for metal removal because of their relatively high costs. Therefore, there is a need to look into alternatives which are effective and economic, and can be used by industries. More practical methods are being explored. One of these methods is to isolate heavy metal-resistant microorganisms, as these have evolved strategies to cope up with stressed conditions (Stadler et al., 2004). Bioremediation of heavy metals using microorganisms has received a great deal of attention in recent years for its potential application in industry, as it is non-destructive and economical (Rehman et al., 2007b; Rise-Roberts, 1998).

Cadmium tolerance and bioaccumulation has been studied in bacteria (Gaballa and Helmann, 2003; Ganguly and Jana, 2002; Hu et al., 2007), cyanobacteria (Inthorn et al., 1996), algae (Feng and Aldrich, 2004) and fungi (Holan and Volesky, 1995; Yan and Viraraghavan, 2003). In the present investigation *C. tropicalis* CBL-1 was found to be resistant to cadmium up to a concentration of 2,800 mg/L. Cadmium-resistant *C. tropicalis* CBL-1 was also found to be resistant to other metal ions and the order of resistance regarding the metal concentration (mM) was Cu(II) > Zn(II) > Ni(II) > Hg(II) > Cr(VI) > Pb(II). 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 the highest metal uptake value obtained was 19.38 mg/g by boiling treated yeast cells. *Candida albicans* and *C. tropicalis* are known for high levels of resistance to the water-soluble ions Hg(II), Pb(II), Cd(II), arsenate (AsO_4^{3-}) and selenite (SeO_3^{2-}) (Berdicevsky et al., 1993). Many *Candida* spp. also

have the capacity to adsorb and/or accumulate metals from their surroundings (Podgorskii et al., 2004).

In the present study *C. tropicalis* CBL-1 showed a fairly high capability to remove metal from the environment. The percent removal of cadmium was 70%. The strain was also capable of decreasing Cd(II) ions (100 mg/L) by 59% and 64% from the medium after 48 and 96 h, respectively. In the same context, Shakoori et al. (2005) reported the potential of CMBLY Pb-2 to remove 62% of Pb(II) within 96 h in a culture medium containing 0.1 mg Pb(II)/L.

The metal uptake, which is an energy-dependent process, was confirmed in the presence of DNP and DCC, which uncouple ATP synthesis from electron transport; they collapse a proton gradient by transporting protons across the membrane. In the presence of metabolic inhibitors, less ATP synthesis takes place and less metal will be uptaken by the organisms. In the present investigation, a significant decrease of total cadmium uptake was observed in the presence of DNP and DCC, which confirms that the main mechanism of metal removal from the medium/environment by *C. tropicalis* CBL-1 is metal accumulation. Both inhibitors decreased total Cd uptake when compared with the control. The decrease of Cd uptake in the presence of DNP and DCC was 28.9% and 17.23%, respectively. The intracellular cadmium accumulation was also greatly inhibited by the presence of inhibitors. The % decrease in Cd-accumulation both in DNP and DCC was 61% and 33%, respectively. This low cadmium-accumulation in the presence of inhibitors indicates that Cd-accumulation requires ATPase activity in *C. tropicalis* CBL-1. Our results are in good agreement with Li et al. (2008).

Accumulation of cadmium in yeast cells was significantly decreased in the presence of both metabolic inhibitors, DNP and DCC, indicating its dependence on ATPase activity. Similar results were also reported by other workers (Li et al., 2008; Malekzadeh et al., 2002). Yeast growth in the presence of metabolic inhibitors indicates metabolism-dependent and metabolism-independent processes of the heavy metal resistance mechanisms.

Rehman et al. (2007a) reported that *C. tropicalis* culture grown in a medium containing Cu(II) (100 mg/L) could reduce 74% of copper from the medium after 96 h of incubation. The yeast was also capable of decreasing Cu(II) ions by 16%, 20%, 29%, 43%, 46%, 55% and 68% from the medium after 6, 12, 18, 24, 30,

48 and 72 h, respectively. *C. tropicalis* was observed to remove 64% of copper from industrial wastewater after 4 days and 74% after 8 days.

In order to assess the ability of the yeast isolate to decrease Cd(II) in contaminated industrial effluents, a mini large-scale experiment was done. Industrial wastewaters harbor a variety of microorganisms including bacteria, fungi, algae and ciliates. They were able to remove only 20 and 30% of cadmium but *C. tropicalis* was able to remove 46 and 60% of cadmium after 6 and 12 days of incubation at room temperature. This 50% greater removal of Cd(II) from the wastewater containing *C. tropicalis* when compared with the original micro-flora present in the industrial effluent after 12 days indicates the extent of *C. tropicalis* survival and removal of Cd(II) from the industrial wastewater. *C. tropicalis* was able to uptake 29 and 42 mg/L of Cd(II) from distilled water after 6 and 12 days, respectively. The amount of Cd-removal from effluent + *C. tropicalis* was higher than that from distilled water + *C. tropicalis* due to the presence of microorganisms in the wastewater. These microorganisms also contribute in removing cadmium ions from the wastewater but these microorganisms were not present in distilled water and the total Cd-removal was done only by *C. tropicalis* (42 mg Cd/L after 12 days) while microorganisms along with *C. tropicalis* were able to remove 60 mg Cd/L after 12 days from the industrial effluent.

Nutrient stress conditions sometimes have a retarding effect on the uptake ability of microorganisms. Sometimes indigenous microorganisms compete with other microorganisms for space and food. No doubt the variations in the physical (pH and temperature) and chemical (different toxic metal ions and complex organic molecules) environment of effluent affect the metal accumulation ability of the micro-flora. In the present study *C. tropicalis* CBL-1 not only exhibited the ability to synergistically survive in contaminated wastewater but also demonstrated a marked increase in remediation of toxic Cd(II) in its presence. However, the yeast isolate under study was able to appreciably decrease the Cd level from the industrial wastewater along with the micro-flora. This affirms the potential role of the *C. tropicalis* CBL-1 in bioremediation of industrial effluents polluted with cadmium. Siloniz et al. (2002b) described the ability of yeast, isolated from sewage sludge, to take up cadmium in response to increasing concentrations of this metal in the culture medium. Moreover Balsalobre et al. (2003) indicated

that both the tolerance to metals and the capacity of the uptake are dependent on ionic metal and yeast species.

Conclusion

C. tropicalis CBL-1 showed high resistance against different heavy metal ions ranging from 1,100 to 3,100 mg/L. *C. tropicalis* CBL-1 could remove Cd(II) 59%, 64% and 70% from the medium after 48, 96 and 144 h, respectively. *C. tropicalis* CBL-1 was also able to remove Cd(II) 46% and 60% from wastewater after 6 and 12 days, respectively. Total and intracellular Cd uptake was decreased in the presence of energy inhibitors. Total Cd uptake in 2,4-dinitrophenol (DNP) and *N,N'*-dicyclohexylcarbodiimide (DCC) was 59.1 and 68.7 mg/L, respectively while intracellular Cd uptake from the medium containing DNP and DCC was 20.5 and 35.2 mg/L, respectively. Cd produced an increase in GSH and NPSH level by 37 and 18% at a 100 mg/L concentration, respectively. *C. tropicalis* CBL-1 accumulated a substantial amount of Cd(II) from the medium (70%) and wastewater (60%) and is adaptable to the local environmental conditions. Therefore *C. tropicalis* CBL-1 may be applicable for the treatment of cadmium-containing wastewater.

Acknowledgments

The technical assistance of Miss Sumbal Zulfiqar and atomic absorption spectrophotometer facilities provided by Dr. A.R. Shakoori (Director, School of Biological Sciences, Pakistan) are gratefully acknowledged.

References

- APHA (1992) Standard Methods for the Examination of Water and Wastewater, 18th ed., APHA, Washington, DC.
- Balsalobre, L., De-Siloniz, M. I., Validerrama, M. J., Benito, T., Larrea, M. T., and Peinado, J. M. (2003) Occurrence of yeasts in municipal wastes and their behaviour in presence of cadmium copper and zinc. *J. Basic Microbiol.*, **43**, 185–193.
- Benson, H. J. (1994) Microbiological applications. Laboratory Manual in General Microbiology, Wan C. Brown Publishers, Dubuque, pp.120–139.
- Berdicevsky, I., Lea, D., Marzbach, D., and Yannai, S. (1993) Susceptibility of different yeast species to environmental toxic metals. *Environ. Pollut.*, **80**, 41–44.
- Blackwell, K. J., Singleton, I., and Tobin, J. M. (1995) Metal cation uptake by yeast: A review. *Appl. Microbiol. Biotechnol.*, **43**, 579–584.
- Brady, D., Glaum, D., and Duncan, J. R. (1994) Copper tolerance in *Saccharomyces cerevisiae*. *Lett. Appl. Microbiol.*, **18**, 245–250.
- Cerbasi, I. H. and Yetis, U. (2001) Biosorption of Ni(II) and Pb (II) by *Phanerochaete chrysosporium* from binary metal system-kinetics. *Water Res.*, **27**, 15–20.
- Delhaize, E., Jackson, P. J., Lujan, L. D., and Robinson, N. J. (1989) Poly(γ -glutamylcysteinyl) glycine synthesis in *Datura innoxia* and binding with cadmium-role in cadmium tolerance. *Plant Physiol.*, **89**, 700–706.
- Feng, D. and Aldrich, C. (2004) Adsorption of heavy metals by biomaterials derived from the marine alga *Ecklonia maxima*. *Hydrometallurgy*, **73**, 1–10.
- Fujs, S., Gazdag, Z., Poljšak, B., Stibilj, V., Milačić, R., Pesti, M., Raspor, P., and Batič, M. (2005) The oxidative stress response of the yeast *Candida intermedia* to copper, zinc, and selenium exposure. *J. Basic Microbiol.*, **45**, 125–135.
- Gaballa, A. and Helmann, J. D. (2003) *Bacillus subtilis* CPx-type ATPases: Characterization of Cd, Zn, Co and Cu efflux systems. *Biometals*, **16**, 497–505.
- Ganguly, S. and Jana, B. B. (2002) Cadmium induced adaptive responses of certain biogeochemical cycling bacteria in an aquatic system. *Water Res.*, **36**, 1667–1676.
- Gharieb, M. M. and Gadd, G. M. (1998) Evidence for the involvement of vacuolar activity in metal(loid)s tolerance: Vacuola-lacking and defective mutants of *Saccharomyces cerevisiae* display higher sensitivity to chromate, telurite and selenite. *Biometals*, **11**, 101–106.
- Gharieb, M. M. and Gadd, G. M. (2004) Role of glutathione in detoxification of metal (loid)s by *Saccharomyces cerevisiae*. *Biometals*, **17**, 183–188.
- Holan, Z. R. and Volesky, B. (1995) Accumulation of Cd, lead and nickel by fungal and wood biosorbents. *Appl. Biochem. Biotechnol.*, **53**, 133–146.
- Hu, Q., Qi, H., Bai, Z., Dou, M., Zeng, J., Zhang, F., and Zhang, H. (2007) Biosorption of cadmium by a Cd²⁺-hyperresistant *Bacillus cereus* strain HQ-1 newly isolated from a lead and zinc mine. *World J. Microbiol. Biotechnol.*, **23**, 971–976.
- Inthorn, D., Nagase, H., Isaji, Y., Hirata, K., and Miyamoto, K. (1996) Removal of Cd from aqueous solution by the filamentous cyanobacterium *Tolypothrix tenuis*. *J. Ferment. Bioeng.*, **82**, 580–584.
- Israr, M., Sahi, S. V., and Jain, J. (2006) Cadmium accumulation and antioxidant responses in the *Sesbania drummondii* callus. *Arch. Environ. Contam. Toxicol.*, **50**, 121–127.
- Jin, Y. H., Clark, A. B., Slebos, R. J., Al-Rafai, H., Taylor, J. A., Kundel, T. A., Resnick, M. A., and Gordenin, A. (2003) Cadmium is a mutagen that acts by inhibiting mismatch repair. *Nat. Genet.*, **34**, 326–329.
- Kapoor, A., Viraraghavan, T., and Cullimore, D. R. (1999) Removal of heavy metals using the fungus *Aspergillus niger*. *Bioresour. Technol.*, **70**, 95–104.

- Kim, H. S., Kwack, S. J., and Lee, B. M. (2005) Alteration of cytochrome P-450 and glutathione S-transferase activity in normal and malignant human stomach. *J. Toxicol. Environ. Health*, **68**, 1611–1620.
- Klaassen, C. D. (2002) Xenobiotic transporters: Another protective mechanism for chemicals. *Int. J. Toxicol.*, **21**, 7–12.
- Kujan, P., Prell, A., Šafar, H., Sobotka, M., Řezankat, T., and Holzer, P. (2005) Removal of copper ions from dilute solutions by *Streptomyces noursei* mycelium. Comparison with yeast biomass. *Folia Microbiol.*, **50**, 309–313.
- Larena, I., Salazar, O., Gonzalez, V., Julian, M. C., and Rubio, V. (1999) Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for ascomycetes. *J. Bacteriol.*, **75**, 187.
- Li, Z., Lu, Y., Zhen, R., Szczypka, M., Thiele, D. J., and Rea, P. A. (1997) A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*; YCF1-catalyzed transport of bis(glutathionato) cadmium. *Proc. Natl. Acad. Sci. USA*, **94**, 42–47.
- Li, Z. and Yuan, H. (2006) Characterization of cadmium removal by *Rhodotorula* sp. Y11. *Appl. Microbiol. Biotechnol.*, **73**, 458–463.
- Li, Z., Yuan, H., and Hu, X. (2008) Cadmium-resistance in growing *Rhodotorula* sp. Y11. *Bioresour. Technol.*, **99**, 1339–1344.
- Malekzadeh, F., Latifi, A. M., Shahamat, M., Levinand, M., and Colwell, R. R. (2002) Effects of selected physical and chemical parameters on uranium uptake by the bacterium *Chryseomonas* MGF-48. *World J. Microbiol. Biotechnol.*, **18**, 599–602.
- Malik, A. and Jaiswal, R. (2000) Metal resistance in *Pseudomonas* strains isolated from soil treated with industrial wastewater. *World J. Microbiol. Biotechnol.*, **16**, 108–117.
- Moran, L. K., Gutteridge, J. M. C., and Quinlan, G.-J. (2001) Thiols in cellular redox signaling and control. *Curr. Med. Chem.*, **8**, 763–772.
- Noctor, G., Gomez, L., Vanacker, H., and Foyer, C. H. (2002) Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signaling. *J. Exp. Bot.*, **53**, 1283–1304.
- Penninckx, M. J. (2002) An overview on glutathione in *Saccharomyces* versus non-conventional yeasts. *FEMS Yeast Res.*, **2**, 295–305.
- Perego, P. and Howell, S. B. (1997) Molecular mechanisms controlling sensitivity to toxic metal ions in yeast. *Toxicol. Appl. Pharmacol.*, **147**, 312–318.
- Pócsi, I., Prade, R. A., and Penninckx, J. (2004) Glutathione, altruistic metabolite in fungi. *Adv. Microb. Physiol.*, **49**, 1–76.
- Podgorskii, V. S., Kasatkina, T. P., and Lozovaia, O. G. (2004) Yeasts biosorbents of heavy metals. *Mikrobiol. Zh.*, **1**, 91–103.
- Rama Rao, V. S., Wilson, C. H., and Maruthi Mohan, P. (1997) Zinc resistance in *Neurospora crassa*. *Biometals*, **10**, 147–156.
- Rehman, A., Farooq, H., and Shakoori, A. R. (2007a) Copper tolerant yeast, *Candida tropicalis*, isolated from industrial effluents: Its potential use in wastewater treatment. *Pakistan J. Zool.*, **39**, 405–412.
- Rehman, A., Shakoori, F. R., and Shakoori, A. R. (2007b) Heavy metal resistant *Distigma proteus* (Euglenophyta) isolated from industrial effluents and its possible role in bioremediation of contaminated wastewaters. *World J. Microbiol. Biotechnol.*, **23**, 753–758.
- Rise-Roberts, E. (1998) Remediation of petroleum contaminated soils. Biological, Physical and Chemical Processes, CRC Press, Boca Raton, Florida.
- Shakoori, A. R. and Qureshi, F. (2000) Cadmium resistant bacteria from industrial effluents and their role in environmental clean-up. *Pakistan J. Zool.*, **32**, 165–178.
- Shakoori, A. R., Zill-i-Huma, Dar, N., and Ali, S. S. (2005) Lead resistant yeast from industrial wastewater capable of decontaminating it of heavy metals. *Pakistan J. Zool.*, **37**, 1–11.
- Siloniz, M., Balsolobre, C., Valderrama, M., and Peinado, J. (2002a) Feasibility of copper uptake by the yeast *Pichia guilliermondii* isolated from sewage sludge. *Res. Microbiol.*, **153**, 173–180.
- Siloniz, M., Payo, E. M., Callejo, M. A., Marquina, D., and Peinado, M. J. (2002b) Environmental adaptation factors of two yeasts isolated from the leachate of a uranium mineral heap. *FEMS Microbiol. Lett.*, **210**, 233–237.
- Singhal, R. K., Anderson, M. E., and Meister, A. (1987) Glutathione, a first line of defense against cadmium toxicity. *FASEB J.*, **1**, 220–223.
- Stadler, N., Lindner, R. A., and Davies, M. J. (2004) Direct detection and quantification of transition metal ions in human atherosclerotic plaques: Evidence for the presence of elevated levels of iron and copper. *Arterioscler. Thromb. Vasc. Biol.*, **24**, 949–954.
- Suh, J. H., Kim, D. S., Yun, J. W., and Song, S. K. (1998) Process of Pb(II) accumulation in *Saccharomyces cerevisiae*. *Biotechnol. Lett.*, **20**, 153–156.
- Villegas, L. B., Amoroso, M. J., and De-Figueroa, L. I. C. (2005) Copper tolerant yeasts isolated from polluted area of Argentina. *J. Basic Microbiol.*, **45**, 381–391.
- Yan, G. and Viraraghavan, T. (2003) Heavy-metal removal from aqueous solution by fungus *Mucor rouxii*. *Water Res.*, **37**, 4486–4496.
- Zafar, S., Aqil, F., and Ahmad, I. (2007) Metal tolerance and bio-sorption potential of filamentous fungi isolated from metal contaminated agricultural soil. *Bioresour. Technol.*, **98**, 2557–2561.