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Heavy Metals Induced Oxidative Stress in Multi-Metal Tolerant Yeast, *Candida* sp. PS33 and its Capability to Uptake Heavy Metals from Wastewater

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ABSTRACT

Metal-resistant yeast strain, *Candida* sp. PS33, isolated from industrial wastewater showed tolerance to Pb (upto 35 mM), As (upto 29 mM), Cu (upto 23 mM), Cd (upto 11 mM) and Cr (upto 8 mM). Exposure to heavy metals at 0.1 mM for 2 days showed a remarkable increase in glutathione (GSH) level after As and Pb treatment in contrast to Cd, Cr and Cu exposure. GSH: GSSG ratio declined after As, Pb and Cr treatment, whereas non-protein thiol levels were higher after Cd and As treatment followed by those of Pb, Cr and Cu treatment. *Candida* sp. PS33 was able to remove 78% (Cd), 70% (As), 82% (Cu), 65% (Cr) and 87% (Pb) from the medium after 8 days of incubation. This multi-resistant yeast can be used efficiently for the removal of toxic metals from the wastewater.

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Authors contribution
AR designed the study and supervised the work. SI performed experiments, analyzed the results and wrote the article. QI helped in statistical analysis.

Key words
Yeast, Glutathione, Oxidative stress, Metal toxicity, Heavy metal uptake.

INTRODUCTION

One of the most important environmental contaminant is heavy metals. Some heavy metals (As, Cd, Hg, Cr) are considered as human carcinogens by International Agency for Research on Cancer (IARC) and are associated with various human diseases (Jomova and Valko, 2011; IARC, 2012). The presence of heavy metals ions in wastewaters constitutes a major environmental and health problem. Measures have to be taken to protect and conserve the environment by reducing the load of heavy metal pollution. Bioremediation of heavy metal ions by using microbial biomass (bacteria, yeast and fungi) is one of the most popular strategies due to environmental friendliness, cost effectiveness and ability to work in a low concentrations of heavy metal ions (Rajendran *et al*., 2003; Yan and Viraraghavan, 2003; He *et al*., 2011; Huang *et al*., 2014). This bioaccumulation arises from metabolism independent extracellular adsorption by surface complexation, ion exchange or electrostatic interaction and from metabolism dependent uptake leading to intracellular accumulation (Gomes *et al*., 2002; Vargas-García *et al*., 2012).

Metals generally generate reactive oxygen species (ROS) directly and increased ROS production can contribute toxicity by disturbing the cell metabolic activities. To cope with increased toxicity, antioxidant enzyme systems in fungi and yeasts get stimulated. The glutathione (GSH)–glutathione disulfide (GSSG) redox system in fungi and yeasts plays a fundamental role in cell homeostasis and is also considered as index of oxidative damage (Valko *et al*., 2007). GSH is involved in many biological processes including DNA synthesis, the metabolic processing of certain endogenous compounds, inactivation/detoxification of xenobiotics and ROS oxidative stress. It also performs numerous functions in protection, transport, and enzymatic catalysis (Penninckx, 2002; Kim *et al*., 2005). Its biological importance is dependent on the redox-active sulfhydryl moiety (-SH) of cysteine residue which can act as a free radical scavenger and modulates protein structure either directly or indirectly by effecting sulfhydryl groups of protein (Klatt and Lamas, 2000).

In the present work, we aim to investigate mechanisms of toxicity associated with the exposure of *Candida* sp. PS33 to multiple-metals (Cd, Pb and Zn), a yeast with potential bioremediation capacity. Specifically, the impact of multi-metals on the yeast glutathione (GSH and GSSG) and non-protein thiols (NPSH) levels were addressed. Additionally, the effect of heavy metals on the total proteins were analyzed by one dimensional gel electrophoresis. Using the information achieved with this work, it was proposed that induction of oxidative

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stress lead to a number of responses occurring during the exposure of the non-conventional yeast *Candida* sp. PS-33 to various metals.

MATERIALS AND METHODS

Media, culturing and handling procedures

Organisms were sampled from industrial effluents and collected in sterilized containers afterwards spread on YPD (2% glucose, 1% yeast extract, 2% bacto-peptone and 2% agar) agar plates (pH 6.5) used for routine cultivation and storage. Liquid minimal salt medium (MSM) containing: 1% glucose, 0.1% $(NH_4)_2SO_4$, 0.015% KH_2PO_4, 0.01% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.0026% $FeSO_4$, 0.0086% $CaCl_2$ (pH±7) was used for all the experiments. Salts of heavy metals ions ($CdCl_2$, $NaAsO_2$, $CuSO_4$, Pb $(NO_3)_2$ and $K_2Cr_2O_7$) were prepared in deionized water and added individually to yeast culture after 24 h incubation period.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) against increasing concentrations of Cd, As, Cu, Pb and Cr was determined. The initial metal concentration used was 0.1 mM prepared from 1 M stock solution. Grown yeast cells were subsequently transferred at a given concentration to next concentration and maximum resistance was evaluated until the yeast strain unable to grow colonies on agar plates. Growth curves, pH and temperature of the yeast isolate were also assessed.

Optimization of growth conditions

The yeast isolate was grown in MSM broth supplemented with various heavy metal (0.1 mM concentration each separately) at 30°C for 48 h. Optical density at 600 nm (OD_{600nm}) was employed as the function of yeast growth. Likewise, for the measurement of optimum pH, the yeast was cultivated in YPD broth of pH 5, 6, 7, 8, and 9. To determine optimum temperature, isolate was grown in YPD broth at different incubating temperatures *viz*., 20°C, 25°C, 30°C, 37°C and 42°C. For both experiment the OD_{600nm} was measured as the function of yeast growth.

Ribotyping

Genomic DNA was isolated according to Masneuf-pomarède *et al*. (2007) and internal transcribed spacer 1 and 5.8S ribosomal RNA gene 5.8S was amplified by polymerase chain reaction (PCR) using two universal oligonucleotide yeast primers, the forward primer (ITS-5; 5-GGAAGTAAAAGTCGTAACAACG-3) and the reverse primer (ITS-4; 5-TCCTCCGCTTATTGATATGC-3) (Larena *et al*., 1999). PCR was preceded at 94°C for 4 min, annealing at 55°C for 2 min, and elongation at 72°C for 10 min. The amplicon of approximately 580 bp obtained was cleaned by the thermo scientific geneJET gel extaction kit method. PCR product was analyzed by agarose gel electrophoresis and sequencing was performed.

Preparation of protein extracts

Cells, grown in MSM containing Cd (0.1 mM), were harvested by centrifugation (1,400 *g*, 10 min) washed twice with phosphate buffer (pH 7). Cells were sonicated for 15 sec with 60 sec interval (5 cycles) and subjected to centrifugation (11,000 x*g*, 10 min) at 4°C. Protein concentration in the samples was measured by method of Bradford with bovine serum albumin as a standard (Bradford, 1976). Protein extracts were stored at -80°C and used for further biochemical analysis.

Quantification of glutathione and cysteine levels

Total glutathione (GSH) and non-protein thiol contents in cell lysates prepared from the yeast cells were estimated by the chemical method as previously described by Israr *et al*. (2006). MSM broth medium (100 mL) was inoculated with 5×10^6/mL of fresh pre-culture yeast cellsand incubated at 30°C with constant agitationon shaker. Glutathione levels were determined following 48 h treatment with oxidants, exponential-phase cells were harvested by centrifugation (4,000 *xg*, 5 min) and collected pellet was washed twice with 5 ml of 1 mM phosphate-buffered saline (pH 7.4) to remove any traces of growth medium, weighed and suspended in 5% sulfosalicyclic acid. Cells were sonicated for 15 sec with 60 sec interval (5 cycles) and centrifuged (1500 *xg*, 10 min) at 4°C.

Quantification of GSH and GSSG levels was done by incubating the reaction mixture contained reaction buffer [0.1M phosphate buffer (pH 7); 0.5 mM EDTA], crude extract or lysate and 3 mM of 5dithio-bis-(2 nitrobenzoic acid) at 30°C for 5 min followed by addition of NADPH (0.4 mM) and 2 µl glutathione reductase (GR) enzyme. Samples were kept at 30°C for 20 min to allow the completion of reaction and absorbance was taken at 412 nm. Glutathione levels in the samples were compared with standard curve constructed by using various concentrations of reduced glutathione.

NPSH level was quantified by mixing100 µl of yeast sample (treated and untreated), 1 mM of 5 dithio-bis-(2-nitrobenzoic acid) and 0.1 M reaction buffer and the reaction was completed by incubating reaction mixture at 30°C for 10 min. A standard curve was prepared from varying concentrations of cysteine to calculate the other non-protein thiol contents in samples. Biomass (%) prepared from oxidant free salt medium was taken as control.

Metals removal potential of yeast strain

Cultures were grown in MSM medium containing 0.1 mM each metal separately, incubated under shaking condition (120 rpm) and aliquots (5 ml) were taken out under sterilized conditions after time interval of 2, 4, 6 and 8 days. The samples were centrifuged at 4,000 *xg* for 10 min, collected culture pellets were weighted and washed thrice with autoclaved distilled water afterwards divided into two parts. One part was washed thrice with 0.1 M EDTA for 10 min. The amount of metal associated with the cell surface was removed as soluble fraction. The second part treated with 0.2 N HNO_3 for acid digestion was left on hot plate for half an hour. Quantitative analysis of metals (Cd, Cr, Cu, and Pb) in the medium or processed by the yeast cells was performed by flame atomic absorption spectroscopy (FAAS) (Zeeman AAS, Z-5000 Model, Hitachi Ltd, Japan) and graphite furnace atomic absorption spectroscopy (GFAAS) (AA Solaar M6 Spectrometer, UK) using air-acetylene burner.

Statistical analysis

Three independent experiments were performed and the data represent the mean (±SD) of four independent experiments. Significance testing between samples was calculated by performing paired Student's t-test. Control group was treated identically but not exposed to any treatment.

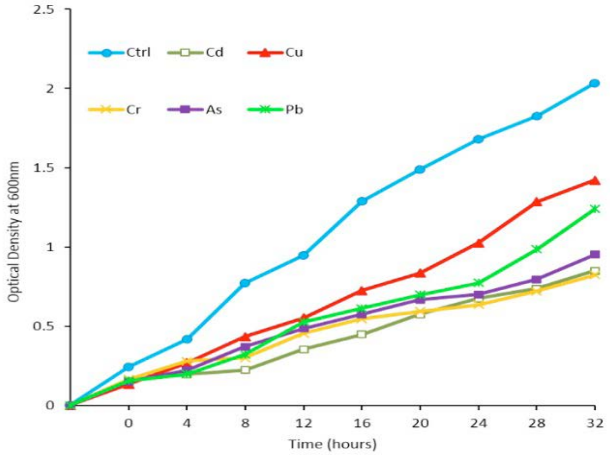

Fig. 1. Growth curve patterns of *Candida* sp. PS33 in the presence of heavy metals (0.1 mM each added separately in the growth medium). Control cells were incubated in the absence of metals for 32 h.

RESULTS

Identification and characterization of yeast isolate

The basic local alignment search tool (http://www.ncbi.nlm.nih.gov/BLAST) analysis of partial nucleotide sequence of the internal transcribed spacer 1 and 5.8S ribosomal RNA gene sequence showed 98% homology to already reported *Candida* sp. The nucleotide sequences coding for *Candida* sp. PS-33 have been submitted to GenBank database under accession number KJ913821.

Candida sp. PS33 grew and survived well at 30°C (pH 7). Its growth was consistently slower versus control in the presence of heavy metals (Cd, As, Cr, Cu, Pb) at a concentration of 0.1 mM (Fig. 1), whereas its biomass content also decreased in the presence of heavy metal ions (Table I). *Candida* sp. PS33 showed metal tolerance value of 35 mM for Pb, 29 mM for As, 23 mM for Cu, 11 mM for Cd, and 8 mM for Cr.

Table I.- Biomass content (%) of *Candida* sp. PS33 grown for 24 h in MSM supplemented with (0.1 mM) and without heavy metals.

Sample	Biomass (%)
Control	100
$CuSO_4$	85 ± 0.15
$Pb(NO_3)_2$	83 ± 2.5
$NaAsO_2$	76 ± 0.25
$K_2Cr_2O_7$	70 ± 0.17
$CdCl_2$	65 ± 3.4

Data are expressed as percentages and values expressed as mean of three values ±SD.

Effect of heavy metals on GSH and NPSH levels

Intracellular GSH pool was enhanced in *Candida* sp. PS33 after exposure to As (49 mM) and Pb (45 mM) while a modest increase was recorded after exposure to Cd (26 mM), Cu (25 mM) and Cr (22 mM) at a concentration of 0.1 mM (Fig. 2). GSH:GSSG ratio declined after Cr (4.4), Pb (2.17) and As (2.0) treatment indicating oxidative stress and higher in Cd (6.3) treated cells as compared to the cells without any metal treatment (Control: 5.1). Interestingly, the ratio remained unaffected with Cu (5.3) treated yeast cells. Likewise, NPSH levels were significantly increased by 74% in Cd, 53% in As, 45% in Pb, 44% in Cu and 33% in Cr (Fig. 3).

Heavy metal removal potential of yeast

Heavy metals removal potential was determined over 8-days exposure. *Candida* sp. PS33 removed 78% Cd, 70% As, 82% Cu, 65% Cr and 87% Pb after 8 days from aqueous medium (Fig. 4).

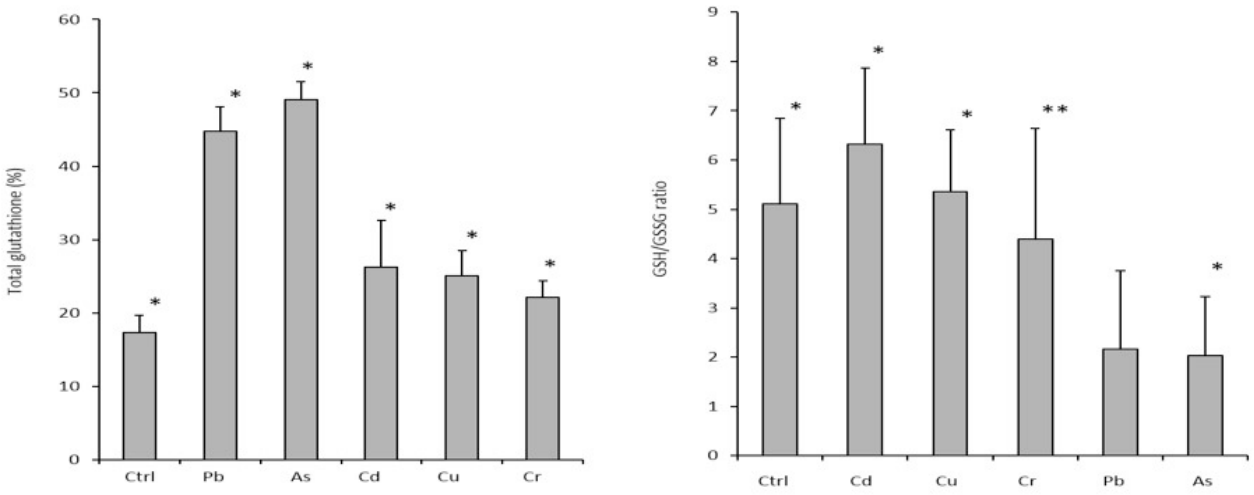

Fig. 2. Total GSH (Left) and GSH/GSSG ratio (Right) in *Candida* sp. PS33. The medium containing 0.1 mM each metal separately was incubated for 48 h. All the experiments were preformed three times. Each point represents the mean (±SD) of four readings.

DISCUSSION

Industrial wastewaters often contain a variety of toxic pollutants, heavy metals such as Cd, Cr, Pb, and As. Removal of these contaminants from the environment by using microbial biomass (bacteria, fungi and yeasts) has been reported by several investigations (He *et al*., 2011; Huang *et al*., 2014; Khan *et al*., 2016). In the present study, a yeast strain showed remarkable multi-metal removing potential from the aqueous medium either by binding of the toxicants to their cell surface or accumulate them within cells to reduce the environmental pollution (Vargas-García *et al*., 2012). The metal ions are cationic in nature and get attached and adsorbed onto the cell wall by electrostatic interactions. Fungi and yeast can accumulate higher concentrations of heavy metal by bioaccumulation and biosorption processes. Bioaccumulation mostly involves initial rapid physical interaction with the cell wall followed by slow transport by various receptors. Once metal get entered it can localized inside specific organelle or sequestered by cys-rich metallothioneins (MTs).

Yeast enzymes can metabolize toxic metal ions by consuming them in their metabolic pathways and exploiting as carbon or energy source (Malik, 2004). The capacity and tolerance of metals uptake is dependent on the metal ionic state as well as the yeast species. Copper resistant strains of *Penicillium janthinellum* and *P. simplicissimum* have also previously been studied (Iskandar *et al*., 2011). *Schizophyllum commune*, *Pycnoporus sanguineus*, *Penicillium* sp., *P. pulmonaris*, *Pichia stiptis*, *Rhizopus arrhizus*, *Trichoderma viride* and *R. mucilaginosa* showed copper adsorption capacities of 1.52, 2.76, 6.2, 15.08, 15.85, 19.0, 19.6 and 26.2 mg/g, respectively (Yahaya *et al*., 2008; Yilmazer and Saracoglu, 2009; Kumar *et al*., 2011).

In order to maintain a reducing environment inside the cells, fungi utilizes antioxidant metabolites such as GSH, NPSH, and MTs (also called stress proteins) which chelate and sequester by metal ions to cope with oxidative stress generated by toxic metal ions. GSH, a most abundant low molecular weight thiol containing free radical scavenger, consisting of three amino acids namely cysteine, glycine and glutamate showed high affinity for metals due to its cysteine residues.

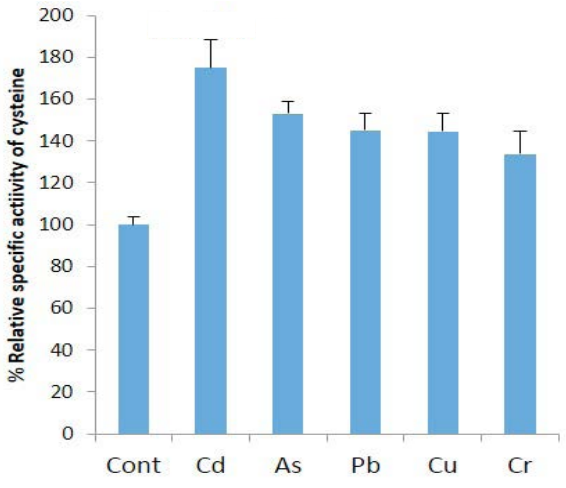

Fig. 3. Specific activity (%) of cysteine and non-protein thiols in *Candida* sp. PS33 exposed to multi-metals (0.1 mM) for 48 h. Values were expressed as means ±SD and experiments were carried out in triplicate.

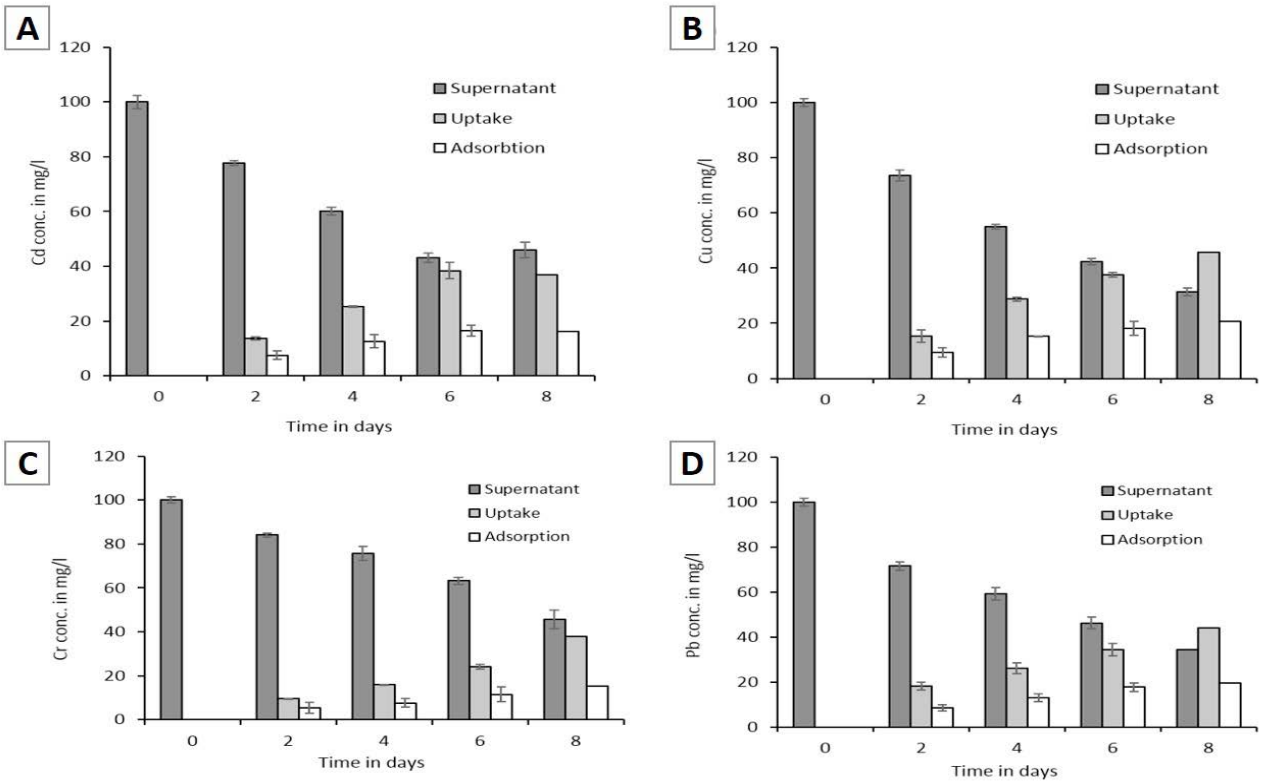

Fig. 4. Bioaccumulation of heavy metals (a) Cd (b) Cu (c) Cr and (d) Pb by *Candida* sp. PS33 in culture medium contaminated with metal ions (0.1 mM each added separately) for 2, 4, 6 and 8 days. Each point represents the mean (±SD) of four readings.

GSH harbors four carboxyl groups and two cysteine residues which can generate octahedral bisglutathionate complexes by interaction with divalent transition metals (Vatamaniuk *et al*., 2000).

Present study revealed an increase in yeast GSH intracellular pool due to oxidative stress generated by heavy metals. GSH provides defense against metal toxicity by detoxification mechanisms that involve binding with metals resulting in complex formation which is a substrate for proteins that mediate vacuolar sequestration. It reacts with (-SH) groups on proteins (protein glutathionylation), thus shielding them from irreversible metal binding and/ or oxidative damage and induces oxidation of metals by altering their oxidation states (Grant *et al*., 1997). The GSH/GSSG ratio was consistently higher in Cd treated cells indicated that GSH profoundly used as an antioxidant by the active cells in agreement with reports of Pe˜na-Llopis *et al*. (2002). In contrast, the ratio was declined in As, Pb and Cr. A decreased GSH:GSSG ratio in arsenic treated *Saccharomyces cerevisiae yap1* mutants have previously been reported by Menezes *et al*. (2008). Reduced or increased GSH redox ratio indicated heavy metal ions had shifted the redox balance of the cell to more oxidized/reduced states which is an extremely sensitive measure for determining the cellular redox status. Under oxidative stress GSH depletion can lead to protein denaturation, potential loss in protein function and aggregation subsequent to protein thiol oxidation (Freeman *et al*., 1997).

Metal binding proteins present in the cytosol such as Cu/Cd binding proteins may also provide a good strategy to detoxify toxic metals. Cysteine and NPSH contents were also strongly induced and enhanced by heavy metals (Ilyas and Rehman, 2015). Cysteine, a sulfur containing amino acid, forms complexes with metal ions in the cytosol and subsequently transported them into the vacuole. This strategy helps in the detoxification of heavy metals. Together, the data obtained indicated that GSH and NPSH are contributed in metal detoxification.

In conclusion, *Candida* sp. PS33 isolated from industrial wastewater, was able to tolerate Pb (35 mM), As (29 mM), Cu (23 mM), Cd (11 mM), and Cr (08 mM). Isolated yeast cells grew and survived well at 30°C (pH 7). Enhanced GSH level was determined in As (49 mM) and Pb (45 mM) treated cells while a modest increase was estimated in the presence of Cd (26 mM), Cu (25 mM) and

Cr (22 mM). Likewise, NPSH levels were significantly increased in Cd (74%), As (53%), Pb (45%), Cu (44%), and Cr (33%). The yeast strain was able to remove 78% (Cd), 70% (As), 82% (Cu), 65% (Cr), and 87% (Pb) after 8 days from aqueous medium. Moreover, its multi metal-resistant nature makes it an effective biosorbent to remove toxic heavy metal ions from the aqueous environment.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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