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A N I N T E R N A T I O N A L J O U R N A L

Oxidative Stress, Chromium-Resistance and Uptake by Fungi: Isolated from Industrial Wastewater

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ABSTRACT

Trichosporon asahii *and* Rhodotorula mucilaginosa *isolated from wastewater effluents were identified as chromium-resistant yeasts. Cr(VI) concentrations at 8 mM and 6 mM were inhibitory for* R. mucilaginosa *and* T. asahii. *Remarkably elevated GSH (69.88 ± 10.01) and GSSG (11.24 ± 0.96) was observed under metal stress in* T. asahii *as compared to* R. mucilaginosa *GSH (18.95 ± 3.19) and GSSG (3.7 ± 2.74) mM g-1* 8 *level. Statistical analysis revealed significantly higher GSH/GSSG ratio in both strains. NPSH (29.84 ± 0.54) level in* T. asahii *was much higher than in* R. mucilaginosa *(6.05 ± 0.24). Chromate reductase (ChR) was assayed and its activity was optimum at 50°C (pH 6) in* T. asahii *while* R. mucilaginosa *showed higher activity at 30°C (pH 7). Activity of both ChRs was enhanced in the presence of Mg, Na, Co and Ca but strongly inhibited by Hg cations. Cr(VI) uptake capabilities were ranged between 43-97% in* R. mucilaginosa *and 35-88% in* T. asahii. *One dimensional electrophoresis revealed enriched bands of cysteine rich metallothioneins suggesting some differential proteins could be overexpressed under Cr(VI) stress.*

Key words: Trichosporon asahii, Rhodotorula mucilaginosa, wastewater, resistance, bioaccumulation

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INTRODUCTION

Chromium (Cr) is the 7th 19 most abundant element on earth that exists in several oxidation states from +2 to +6 with an average concentration of 100 ppm [1]. Cr is widely used in chrome electroplating and finishing, leather tanning, textile dyeing, stainless steel welding, ferrochrome production, metal processing industries, corrosion inhibition in power plants, wood treatment, mining equipment, manufacturing of refractory materials, pigments, and in nuclear facilities [2,3]. Cr is regarded as a priority pollutant by the USEPA [4] which poses threat to humans and has been linked to genotoxicity, carcinogenicity, allergenicity and mutagenicity [5-7]. Industrial wastewaters contain both chromium and salt ions which have toxic effects on the microbial consortia [8]. Stable forms of chromium in nature can be either trivalent or hexavalent [9]. Hexavalent chromium compounds are comparatively more toxic than trivalent owing to higher solubility, rapid permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acids [10,11]. Trivalent chromium, an essential trace element, plays an important role in regulating fat and glucose metabolism and involve in proper functioning of insulin [12] in all living organisms. Cr(VI) is reduced partially to highly unstable Cr(V) radical inside the cells that generates oxidative stress through the production of reactive oxygen species (ROS), leading to carcinogenicity [13].

Microbial tolerance and reduction of Cr(VI) to Cr (III) is considered to be an effective and independent phenomena [14] of combating Cr(VI) pollution. Yeast biomass has the ability to accumulate variety of heavy metals to varying degrees under a wide range of external conditions [15]. Cr(VI) gets entered into the cell through non-specific sulfate transporters [16] by facilitated diffusion and a gradient between the two sides of the cell membrane is established by the metabolically active cells, which constantly reduce Cr(VI) to Cr(III) enzymatically (flavoenzymes) and non-enzymatically [glutathione (GSH), NADPH and ascorbate] [17]. Yeast's response to chromium involves various cellular processes such as redox reactions [18], interactions with cellular organelles, binding by cytosolic molecules [19], as well as formation of protein-DNA and Cr-DNA adducts, DNA strand breaks and DNA–DNA cross links [20,21]. Due to oxidative stress produced by Cr, induction of stress proteins, Cr entrapment into membranous capsules, metal precipitation, chelation and active efflux were observed in other organisms [14], or were hypothesized to occur in yeast [22]. However, the detailed mechanisms regarding yeast-Cr interactions are yet unclear. In this study, Cr(VI) resistant yeasts, isolated from wastewater effluents were exposed to 100 mg/L Cr(VI). Glutathione and NPSH contents in metal treated and untreated samples were also investigated. Chromate reductases (ChRs) involved in Cr(VI) reduction were assayed. The amount of chromium associated with cell surface, accumulated inside cell or present in the medium were assessed by atomic absorption spectrometer (AAS). Protein profiling was also studied by one dimentional (1DE) gel electrophoresis.

MATERIALS AND METHODS

Isolation, Screening and Identification

Wastewater samples were collected from the synthetic mills and industrial effluents of Sheikhupura, located near to Lahore and their physiochemical parameters *viz.*, pH and temperature were noted. Cultures were maintained on Yeast Potato Dextrose (YPD) agar plates comprised of glucose 20 g/L, peptone 20/g L, yeast extract 10/g L and agar 20/g L. Isolation was done by spreading 100 mL of wastewater samples on

YPD agar plates. For screening, yeast isolates were aerobically grown in salt medium containing: 10 g/L glucose, 1 g/L (NH4)2SO4, 0.15 g/L KH2PO4, 0.1 g/L K2HPO4, 0.1 g/L MgSO4·7H2O, 0.026 g/L FeSO4 and 0.086 g/L CaCl2. The pH of the medium was adjusted to 7.0-7.2; medium was sterilized at 121°C for 20 min, inoculated with yeast culture. The isolated strains were evaluated for their tolerance to Cr(VI) ions at different concentrations and highest resistance strains were selected for further experiments. Controls were treated identically but without heavy metal exposure.

DNA was isolated and polymerase chain reaction (PCR) was performed by 35 cycles of denaturation at 94°C for 4 min, annealing at 55°C for 2 min, and elongation at 72°C for 10 min. PCR reaction mixture contained 3 µL PCR buffer, 3 µL dNTPs, 2.5 µL of each forward and reverse primer, 0.5 µL taq polymerase, 2.5 µL MgCl2, 5 µL nuclease free water and 6 µL genomic DNA. The 18S ribosomal RNA gene was cleaned by the thermo scientific geneJET gel extraction kit method and sequencing was performed.

Survival to Heavy Metal Exposure

Minimum inhibitory concentration was assessed by growing cultures on YPD agar plates with increasing concentrations of K2CrO7 [23]. Grown yeast cells were subsequently transferred at a given concentration to next concentration and maximum resistance was evaluated until *T. asahii* and *R. mucilaginosa* cells were unable to grow as colonies on metal-containing agar plates. Any color changes of cultures in response to metal exposure were carefully noted.

Quantification of GSH and NPSH

Analysis of total glutathione (GSH) and non-protein thiol (NPSH) contents in cell lysates was estimated by the chemical method described by Israr et al. [24]. MSM broth medium (100 mL) was taken in 250 Ml flask labelled as control and treatment and inoculated with 5×106/mL of fresh pre-culture yeast cells followed by incubation at 30^{o}C with constant agitation on shaker (120 rpm). Cells were centrifuged at 1,400 *g* for 10 min and washed twice with 1 mM phosphate-buffered saline (pH 7) to remove any traces of growth medium, weighed and suspended in 5% (w/v) sulfosalicylic acid. Pellet was sonicated for 15 sec with 60 sec interval (5 cycles) and centrifuged at 11,000 *g* at 4^{o}C for 10 min. The lysate obtained was used to determine GSH and NPSH levels.

Quantification of GSH and GSSG levels was done by incubating the reaction contained reaction buffer (0.1M phosphate buffer of pH 7) and 0.5 mM EDTA), crude extract and 3 mM of 5 dithio-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 further 20 min to allow the reaction to complete and absorbance was taken at 412 nm by UV–vis spectrophotometer (Hitachi U-2800, Tokyo, Japan).

Glutathione level in the samples were compared with standard curve constructed by using various concentrations of reduced glutathione (GSH). GSH levels in the samples are expressed in mM/g of cells.

Biomass prepared from oxidant free salt medium was taken as control.

NPSH were quantified by mixing 100 µL of extracted sample (treated and untreated), 1 mM of 5 dithio bis-(2-nitrobenzoic acid) and reaction buffer containing 0.1 M phosphate buffer (pH 7) and 0.5 mM EDTA. The reaction was completed by incubating reaction mixture at 30°C for 10 min. A standard curve was prepared from varying concentrations of cysteine. Control was identically treated without exposure of heavy metal ions.

Determination of Intracellular Enzyme Activity

Intracellular chromate reductase activity was estimated by collecting the cell pellet after centrifugation (1,500 *g* for 10 min.) of culture, washing twice with sodium phosphate buffer (50 mM, pH 7.0) and lysing by sonication for 15 sec with a 60 sec interval (5 cycles) at 4°C. This sonicated suspension or cell free extract (CFE) was collected by centrifugation (4,000 *g*) for 10 min and used as a crude enzyme source for activity analysis. The protein concentration was determined by Bradford [25] assay using bovine serum albumin (BSA) as a standard.

ChR activity was determined by means of an enzyme assay comprised of crude enzyme, 0.1 mM NADH and 20 µM Cr(VI) in 50 mM potassium phosphate buffer of pH 6.0. The reaction was initiated by adding freshly prepared NADH as an electron donor and incubating the reaction mixture at 30°C for 30 min [26] and percentage relative activity of crude enzyme was calculated. Assay mixtures containing no enzyme or NADH were used as respective controls. One unit of chromate reductase activity is defined as the amount of enzyme that reduced one µmole of Cr(VI) per min per ml under the assay conditions at 30°C.

Reduction of Hexavalent Chromium

Reduction was estimated by measuring the decrease in hexavalent chromium in the culture filtrate by diphenylcarbazide (DPC) method [27]. The reaction mixture was kept at room temperature for 10 min for pink-violet colored complex formation in acidic solution and optical density was taken at 540 nm.

Effect of Temperature, pH and Metal Ions

Chromate reductase was checked at selected temperatures of 30, 40, 50, 70 and 90°C by incubating the enzyme reaction mixture by the standard enzyme assay method. The pH profile of crude enzyme was determined by incubating enzyme over a pH range of 5-9 while keeping the reaction mixture at 30°C for 30 min. For pH profile, buffer systems used were sodium acetate buffer; pH (5.0-6.0), sodium phosphate buffer; pH (7.0-8.0), and Tris-HCl buffer; pH (9.0). Reaction mixture without enzyme (control) was prepared under the same condition and was used to measure the possible changes in O.D. Different chloride metal salts in the form of NaCl, MgCl2, CoCl2, HgCl2 and CaCl2 were selected to assess their effects on enzyme activity. A reaction mixture with no metal ion added in reaction was taken as control.

Chromium Uptake Processes by Yeasts

Cultures were grown in MSM medium, incubated under shaking condition (120 rpm) and aliquots (5 mL) were taken out under sterilized conditions after time interval of 2, 4, 6, 8, 10, and 12 days. The samples were centrifuged at 4,000 *g* 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 0.1M EDTA for 10 min. The amount of metal associated with the cell surface was removed as soluble fraction.

The second part [(acid digested; 0.2 N HNO3, H2SO4 (1:1)] was left on hot plate for half an hour. Total chromium content present in the medium or processed by the yeast cells was estimated by atomic absorption spectrometer (Zeeman AAS, Z-5000 Model, Hitachi Ltd, Japan) using flame (air-acetylene burner) at 359.3 nm [28,29].

Metallothioneins Profiling

One dimentional (1-DE) gel electrophoreses was performed on 14% polyacrylamide gels. Protein samples (20 µg/µL) were precipitated with an equal volume of 10% chilled TCA (v/v) and centrifuged at 11,000 g for 15 min. Proteins were electrophoresed at a constant voltage (120 V) according to Laemmli [30].

Statistical Analysis

Three independent experiments were performed and data shown in this article was average values of means and ± standard deviation (SD). Significance testing between samples was calculated by performing Student's *t*-test and analysis was done with the program statistical package for social sciences (SPSS) version 15. Control group was treated identically without exposure to any treatment.

RESULTS AND DISCUSSION

Yeasts isolated from metal polluted wastewater had a potential to survive well in heavy metal ions. A total of 16 yeasts were isolated and two of them were selected on the basis of higher chromium tolerance.

Both strains exhibiting higher Cr(VI) resistance were subjected to molecular identification. The nucleotide sequence similarities were determined using the basic local alignment search tool (http://www.ncbi.nlm.nih.gov/BLAST). Yeasts were identified as *Rhodotorula mucilaginosa* and *Trichosporon asahii* under accession number JQ966756 and KJ 913820.

Minimum Inhibitory Concentration (MIC) and Color Change

R. mucilaginosa and *T. asahii* showed maximum resistant to Cr(VI) up to 8 mM and 6 mM, respectively. Recently, *T. asahii* can tolerate NaAsO2 and CdCl2 up to 30 mM and 10 mM, respectively [23]. *C. tropicalis* can tolerate Cr(VI) up to 5 mM [31] in accord with our results. Wang et al. [32] stated that *Rhodotorula* sp. Y11 isolated from mine soil could survive 2000 mg/L cadmium. Biomass (Table 1) content in both strains was reduced as compared to the controls. The color of *T. asahii* mycelium and red yeast was affected by Cr(VI) and transformed to green with time course in comparison with the control simply due to the reduction or transformation of Cr(VI) in solution (Fig. 1). This color change may be due to yeast cells adapt and synthesize essential enzymes required for the accumulation and reduction of Cr(VI) to Cr(III).

Table 1. Gram fresh weight (biomass) of *R. mucilaginosa* and *T. asahii* grown in MSM supplemented with and without chromium.

Sewage fungal isolates	Fresh weight expressed as percentage (%)			
	Sample 1	Sample 2	Sample 3	Mean ± SD
R. mucilaginosa				
Control (without heavy metal)	100	100	100	100
Medium with $K_2Cr_2O_7$	81	89	89	86 ± 4.77
T. asahii				
Control (without heavy metal)	100	100	100	100
Medium with $K_2Cr_2O_7$	80	79	76	78 ± 7.32

Three biological replicates were used and an equal gram weight was taken for protein extraction.

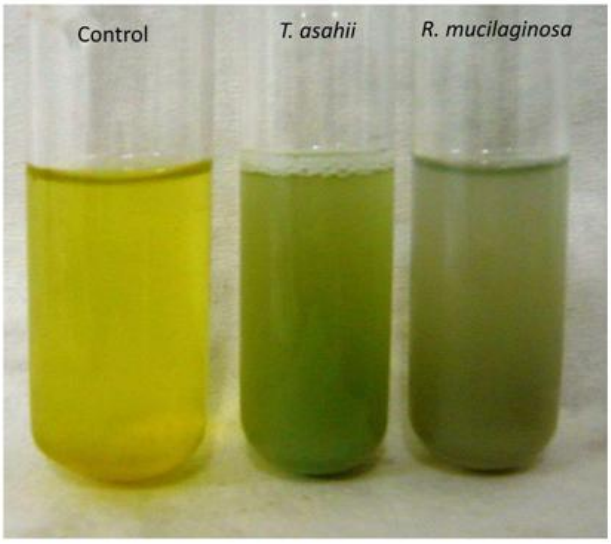


Fig 1 Hexavalent chromium reduction by *R. mucilaginosa* and *T. asahii* cells

Growth Curves

Slow growth pattern of *R. mucilaginosa* and *T. asahii* strains was observed under Cr(VI) stress. Growth rates (Fig. 2a, b) of both isolates were extended as compared to controls. *C. intermedia* showed extended lag growth phase at 50 mg/L. Ilyas and Rehman [31] also observed extended lag phase of *C. tropicalis* cultures grown in medium containing heavy metal ions.

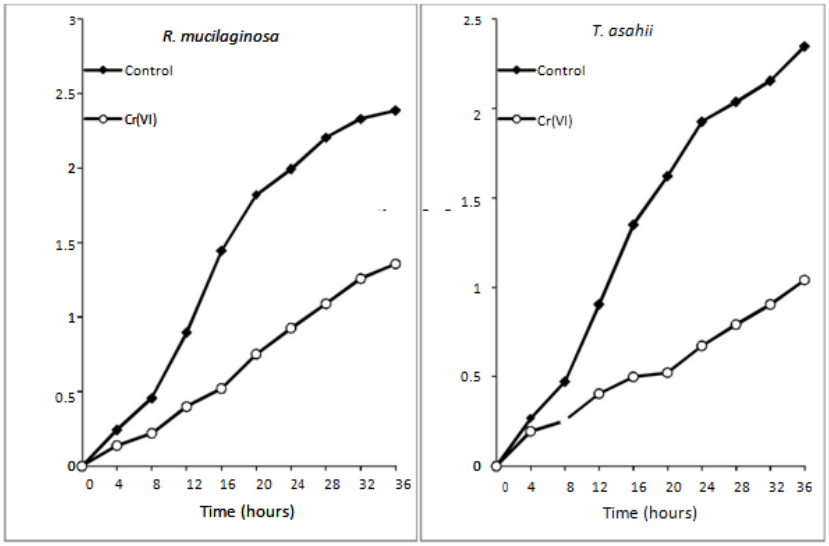


Fig 2 Growth curves for *R. mucilaginosa* and *T. asahii* with and without Cr(VI)

Quantification of GSH and NPSH

Heavy metal exposure had resulted in increased GSH intracellular pool and this accumulation eventually transformed GSH/GSSG ratio. Remarkably elevated GSH (69.88 ± 10.01) and GSSG (11.24 ± 0.96) was observed under metal stress in *T. asahii* as compared to GSH (18.95 ± 3.19) and GSSG (3.7 ± 2.74) mM/g FW in *R. mucilaginosa* (Table 2). Taking into account the total GSH concentration, more than two fold growths was observed under K2Cr2O7 stress in *T. asahii* as compared to *R. mucilaginosa* (Fig. 2a). The rise in regeneration of GSH in response to oxidative stress could be a significant determinant of cell survival. Previous studies have been revealed that increasing GSH synthesis through overexpression of *GSH1* augments

the GSH pool by 50–66% [33] and various metabolic processes lead to induction of enzymes involved in the GSH pathway [34]. GSSG, a potential toxicant, was detected at a very low concentration in normal cells than in stressed cells. GSH/GSSG ratio was also higher in much in *T. asahii* than in *R. mucilaginosa* K2Cr2O7 treated cells in agreement with reports of Peña-Llopis et al. [35]. Nonprotein thiol (NPSH) level was increased to 29.84 ± 0.54 in *T. asahii* and 6.05 ± 0.24 in *R. mucilaginosa* (Table 2). Recently, Ilyas and Rehman [31] also reported an increased NPSH contents in Cr(VI) treated *C. tropicalis*.

Table 2. Intracellular levels of GSH, GSSG, total glutathione, GSH/GSSG ratio and NPSH in *R. mucilaginosa* and *T. asahii* with and without chromium.

Yeast isolates	GSH (mM/g FW)	GSSG (mM/g FW)	GSH+GSSG (mM/g FW)	GSH/GSSG	NPSH (mM/g FW)
T. asahii without $K_2Cr_2O_7$	36.83 ± 2.12	7.0 ± 4.13	43.83 ± 6.25	5.26 ± 10.24	13.0 ± 0.013
T. asahii with $K_2Cr_2O_7$	69.88 ± 10.01	11.24 ± 0.96	81.12 ± 10.97	6.21 ± 22.54	29.84 ± 0.54
R. mucilaginosa without $K_2Cr_2O_7$	7.67 ± 0.95	1.77 ± 0.36	9.46 ± 1.31	4.32 ± 2.5	3.0 ± 0.08
R. mucilaginosa with $K_2Cr_2O_7$	18.95 ± 3.19	3.7 ± 2.74	22.65 ± 5.93	5.12 ± 8.74	6.05 ± 0.24

Experiments were performed in triplicate (n=3) and p<0.1where values were expressed as mean ± SE.

Chromium Reductase Assay

Both the strains raised and induced the intracellular chromate reductase (ChR) activity by reducing Cr(VI) present in the culture medium. *R. mucilaginosa* showed 0.5 fold whereas *T. asahii* exhibited 0.35 fold increase in ChR activity when compared to controls (Fig. 3). Das and Chandra [36] noticed an increase in chromate reductase activity when working with Cr(VI) *Streptomyces* sp. M3 cultures. The enzymatic conversion of Cr(VI) to Cr(III) was reported in *Candida maltose* [37], *Candida utilis* [18] and fungi such as *Hypocrea tawa* [38] and *Aspergillus* sp. [39]. Microbes of the genera *Pseudomonas*, *Arthrobacter*, *Escherichia* and *Bacillus* have been reported to reduce Cr(VI) through soluble chromate reductase [40,41]. Negligible reduction was observed in controls (Fig. 1) indicating the role of yeast's enzymes in Cr (VI) reduction [42,43].

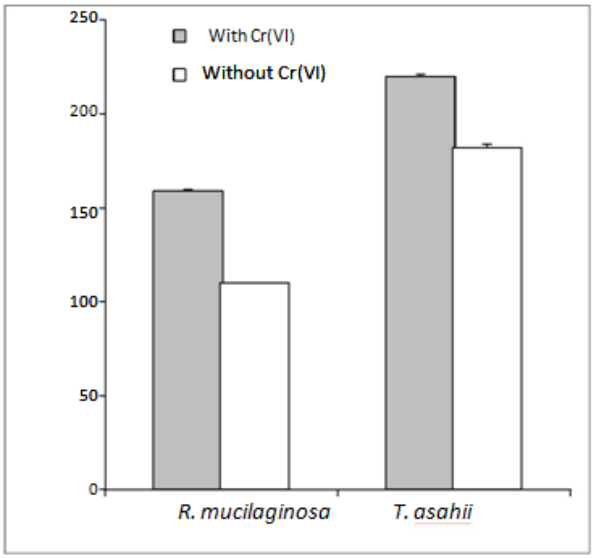
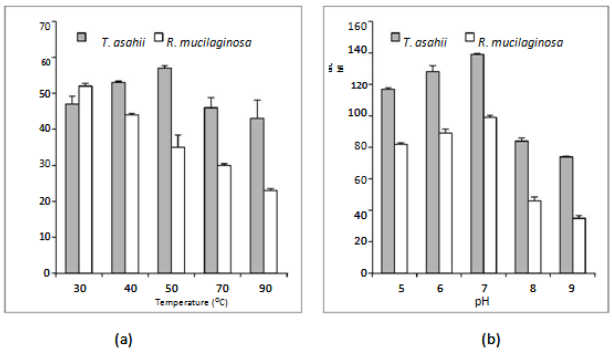


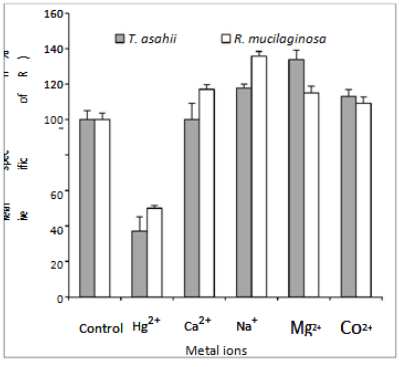
Fig 3 Relative specific activity of chromate reductase in *R. mucilaginosa* and *T. asahii* Values were expressed as mean of ± SD where *p=0.05 and **p=0.1 and all experiments were performed in triplicate

Effect of Temperature, pH and Metal Ions on Enzyme Activity

Effect of temperature in the range of 30-90°C was evaluated. ChR from *T. asahii* exhibited maximum activity at 50o192 C while *R. mucilaginosa* showed higher chromate reductase at 30°C (Fig. 4a). Maximum chromate reductase activity observed by *Pichia jadinii* M9 and *Pichia anomala* M10 was 60 and 50°C, respectively [44]. Among bacterial ChRs, the optimal temperature varies in the range 30-50°C [45,46].



(a) (b)



(c)

Fig 4 Effects of temperature (a) pH (b) and metal ions (c) on chromate reductase activity in *R. mucilaginosa* and *T. asahii*. Values were expressed as mean of ± SD where *p=0.05 and **p=0.1 and all experiments were performed in triplicate.

pH is a major factor affecting efficiency of enzymes as acidic or strongly alkaline pH inactivates enzymes. Experiments were also conducted to elucidate whether pH affected chromate reductase activity and it was assessed that enzymatic activity was greatly affected changed due to pH. Chromate reductase was moderately stable at pH range from 5-6 in *R. mucilaginosa*; nevertheless its optimum pH was 7 in sodium phosphate buffer (Fig. 4b). The ChR of *T. asahii* had a higher activity in sodium acetate buffer at pH 6 which correlates with reports of Martorell et al. [44]. Chromate reductase activity was lost to 55% in *T. asahii* and 53% in *R. mucilaginosa* with increasing pH suggesting a decline in chromate reductase activity resulted with increasing pH values. At pH values above or below the optimum, a decline in enzyme activity was pronounced.

The presence of heavy metal ions in industrial effluents could potentially inhibit or enhance chromate reductase activity. The enzyme activity was also sensitive to metal ions (in buffer) and it was found that Na+ enhanced enzyme activity to 36% while Ca2+ Mg2+, and Co2+ activated the enzyme more modestly (Fig. 4c) in *R. mucilaginosa.* Mg2+ and Ca2+ enhanced chromate reductase in *T. asahii* to 34 and 17%, respectively. Both strains showed negligible increase in enzyme activity on Co2+ addition. In this study relative chromate reductase activity was greatest when treated with Na+ and Mg2+ but, interestingly, other divalent metal ions also showed increased enzyme activity suggesting the enzyme is not absolutely specific for Na+ or Mg2+. Inhibition of enzyme activity by Hg ions was noticed which was higher (63%) in *T. asahii* and reduced to (50%) in *R. mucilaginosa* (Fig. 4c). These results agree with reports of Camargo et al. [47] and Elangovan et al. [46].

Metal Uptake Potential of Yeasts

Cr(VI) in the medium interact continuously with cell wall surface of the *R. mucilaginosa* yeast cells adsorbing 11.21, 14.81, 19.35, 23.07, 25.05 and 27.04 and accumulated 32.02, 45.25, 56.21 62.58, 66.94, and 70.07 mg/g efficiently after 2, 4, 6, 8, 10 and 12 days (Table 3). Chromium removal efficiency was ranged between 43-97% (Fig. 5a) within 2 and 12 days suggesting the metal in the medium is processed enzymatically. *T. asahii* cells adsorbed 7.34, 11.05, 13.22, 16.28, 18.29, 21.01 mg/g 219 and taken up 28.05, 34.21, 45.25, 55.09, 64.57 and 67.14 mg/g within 2 and 12 days of incubation (Table 3). The metal removal potential was 35-88% in *T. asahii* (Fig. 5b). These results clearly indicated that both strains are good candidates for accumulating and removing chromium from the environment although the metal removal efficiency of *R. mucilaginosa* is higher than that of *T. asahii*. Li and Yuan [48] reported maximal metal uptake value of 19.38 mg/g by *Rhodotorula* sp. Y11. The highest chromium adsorption by *R. mucilaginosa* and *T. asahii* indicated more binding sites on cell wall as well as their potential use as bio sorbent and effectiveness to remove Cr(VI) ions from wastewater. Yeasts can accumulate higher concentration of heavy metal ions by bioaccumulation process rather than biosorption. Yeast and fungal enzymes incorporate the toxic heavy metal ions by consuming them in their metabolic pathways and exploiting as carbon or energy source [49].

Table 3. Amounts of heavy metal ion chromium taken up and adsorbed by *R. mucilaginosa* and *T. asahii*.

Time	**Uptake (mg/g)**		**Adsorption (mg/g)**	
(days)	***R. mucilaginosa***	***T. asahii***	***R. mucilaginosa***	***T. asahii***
2	32.02 ± 3.12	28.05 ± 8.24	11.21 ± 2.85	7.34 ± 4.25
4	45.25 ± 5.07	34.21 ± 3.91	14.81 ± 0.51	11.05 ± 0.07

6	56.21 ± 13.05	45.25 ± 9.01	19.35 ± 20.16	13.22 ± 4.14
8	62.58 ± 0.21	55.09 ± 0.33	23.07 ± 1.21	16.28 ± 6.89
10	66.94 ± 2.25	64.57 ± 1.62	25.05 ± 12.84	18.29 ± 0.65
12	70.07 ± 0.21	67.14 ± 15.41	27.04 ± 0.25	21.01 ± 13.27

Experiments were performed in triplicate (n=3) and p<0.1where values were expressed as mean ± SE.

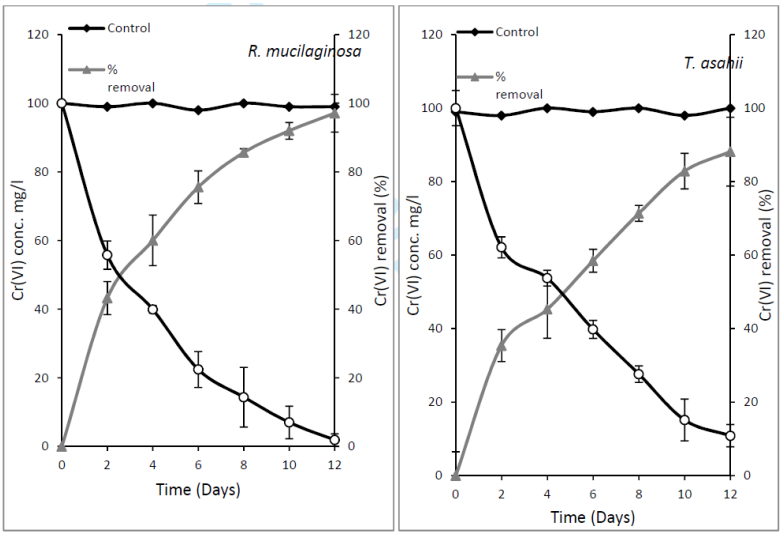


Fig 5 Estimated concentrations of chromium taken up and adsorbed by *R. mucilaginosa* and *T. asahii* yeast cells. Metal in the samples was assayed for metal estimation by using atomic absorption spectrophotometer (AAS). Controls contained heavy metal ions but did not contain yeast cells.

The heavy metals (Cd, Cu, Pb) uptake by consortium of fungi, *Penicillium* sp. A1 and *Fusarium* sp. A19 was significantly higher as revealed by Pan et al. [50]. The maximum biosorption capacity of macro fungus, *Amanita rubescens* determined was 27.3 mg/g for Cd [51]. Engineered *Saccharromyces cerevisiae* cells have been shown enhanced arsenite accumulation by overexpression of transporters responsible for metal influx [52]. Biosorption and uptake are mainly used to treat water containing variety of metal ions although these processes may be influenced and affected by the presence of other metal ions. The bioaccumulation processes revealed by yeasts are inexpensive and environment friendly methodology to remove dissolved heavy metal ions from the wastewater before its use in agriculture for irrigation purposes or discharge into the water bodies.

Metallothioneins Profiling

Expression of proteins in metal treated and untreated cultures were explored by one dimentional (1-DE) gel electrophoresis. Known protein concentration (20 µg/µL) was used and most of the differentially expressed protein bands were seen in yeast cultures exposed to chromium. Chromium treated *R. mucilaginosa* showed increased intensity of 16 and 22 kDa bands which were much weaker in control and some additional proteins were also induced under stress conditions (Fig. 6). Likewise, in *T. asahii*, stronger 45 and 30 kDa protein bands were induced by Cr (Fig. 8). Protein banding patterns revealed different intensities between control and treatments although overall protein loadings were equivalent.

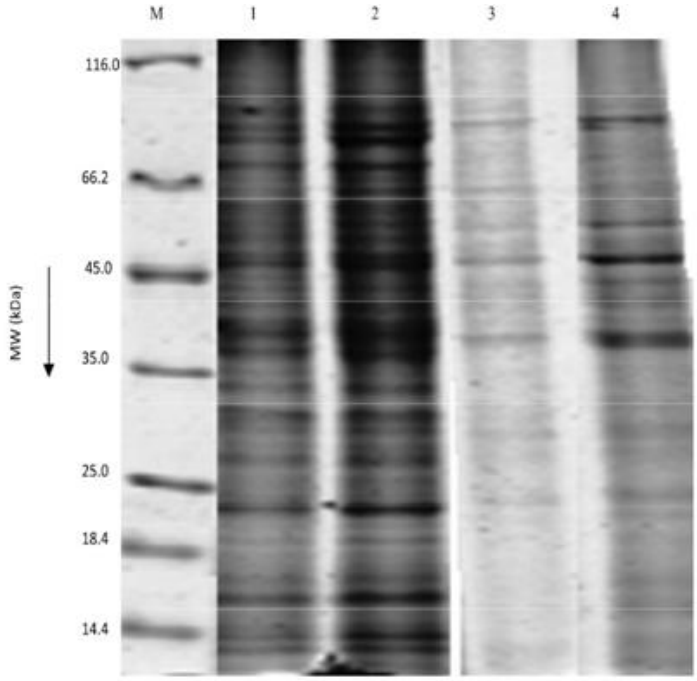


Fig 6 SDS-PAGE analysis of total proteins extracted from *R. mucilaginosa* and *T. asahii* stained with Coomassie blue R-250. Lane M, protein maker; lane 1 and 2 are showing control and Cr treated *R. mucilaginosa*; and lane 3 and 4 illustrate control and Cr treated *T. asahii*, respectively

Low molecular weight metallothioneins (MTs) were abundant in all chromium treated samples implying the possible role of in protection and survival of fungi against metal oxidative stress. Certain bands appeared in treatment were absent in control, especially lower molecular weight proteins in the mass range of <20 kDa with increased intensity observed in metal-treated samples.

CONCLUSION

In conclusion, wastewater yeasts, *R. mucilaginosa* and *T. asahii*, showed considerable resistance towards hexavalent chromium. Remarkably elevated GSH and GSH/GSSG ratio was observed under metal stress in *T. asahii* as compared to *R. mucilaginosa*. NPSH contents were also enhanced under Cr(VI) stress.
Chromium reductases (ChRs) isolated exhibit maximum activity at 30°C (pH 7) in *R. mucilaginosa* and 50°C (pH 6) in *T. asahii*. The activity pattern was not affected to higher extent in the presence of metal ions. Cr (VI) was absorbed and adsorbed by the yeast cells and reduced to Cr(III) by the chromate reductase (ChR). Certain bands present in controls were absent in treatments and the production of low molecular weight MTs were abundant in metal induced samples. Current investigation clearly demonstrated that yeast strains isolated have a strong potential to reduce toxic and soluble Cr (VI) to the less toxic and insoluble Cr(III) and hence can be employed as a biosorbant for Cr(VI) detoxification from the contaminated effluents

COMPETING INTERESTS

We declare that we have no competing interests.

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