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SIDRA ILYAS[1], DILARA A. BUKHARI[2], ABDUL REHMAN[1]

CHROMIUM(VI) UPTAKE AND RESISTANCE BY *P. KUDRIAVZEVII* AM-4 AND *CANDIDA* SP. PSM-33 AND THEIR POTENTIAL USE IN WASTEWATER TREATMENT PLANTS

Two yeast strains already identified as *Pichia kudriavzevii* AM-4 and *Candida* sp. PSM-33 were able to resist Cr(VI) up to 400 and 350 mg/dm^3, respectively. The stability and optimum temperature of chromate reductase in both yeast strains was maximal at 30 °C. *Candida* sp. PSM-33 showed the higher chromate reductase activity at pH 5 whereas *P. kudriavzevii* AM-4 exhibited maximum activity at pH 7. Both chromate reductases (ChRs) activities were enhanced in the presence of Mg, Na, Co, and Ca but strongly inhibited by Hg cations. The total cell Cr(VI) uptake capabilities were 15–68 mg/g in *Candida* sp. PSM-33 and 17–73 mg/g in *P. kudriavzevii* AM-4 within 2–12 days of growth. It was found that 23–94% of Cr(VI) reduction was achieved by *P. kudriavzevii* AM-4 while *Candida* sp. PSM-33 showed 21–88% reduction at a concentration of 100 mg/dm^3. Proteins extracted from *P. kudriavzevii* AM-4 and *Candida* sp. PSM-33 followed by one-dimensional electrophoresis revealed enriched bands of low molecular-weight metallothioneins (MTs) suggesting some differential proteins could be expressed under Cr(VI) treatment. Both yeast strains can be used to ameliorate the wastewater contaminated with toxic metal ions.

1. INTRODUCTION

Chromium (Cr) is the 7th most abundant element on Earth that exists in several oxidation states from 2+ to 6+ with an average concentration of 100 mg/dm^3. Chromium 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 [1]. Chromium is regarded as a priority

[1]Department of Microbiology and Molecular Genetics, University of the Punjab, New Campus, Lahore 54590, Pakistan, corresponding author A. Rehman, email address: rehman_mmg@yahoo.com

[2]Department of Zoology, GC University-Lahore, Pakistan.

pollutant that poses threat to humans and has been linked to genotoxicity, carcinogenicity, allergenicity, and mutagenicity [2]. Industrial wastewaters contain both chromium and salt ions which have toxic effects on the microbial consortia [3]. Stable forms of chromium in nature can be either trivalent or hexavalent [4]. Hexavalent chromium compounds are comparatively more toxic than trivalent ones owing to higher solubility, rapid permeability through biological membranes, and subsequent interaction with intracellular proteins and nucleic acids [5]. Trivalent chromium, an essential trace element, plays an important role in regulating fat and glucose metabolism and is involved in the proper functioning of insulin [6] in all living organisms. Cr(VI) is reduced partially to highly unstable Cr(V) radicals inside the cells that generate oxidative stress through the production of reactive oxygen species (ROS), leading to carcinogenicity [7].

Microbial tolerance and reduction of Cr(VI) to Cr (III) is considered to be an effective and independent phenomenon [8] of combating Cr(VI) pollution. Yeast biomass can accumulate a variety of heavy metals to varying degrees under a wide range of external conditions [9]. Cr(VI) gets entered into the cell through non-specific sulfate transporters [10] 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). Yeast's responses to chromium involve various cellular processes such as redox reactions, interactions with cellular organelles, binding by cytosolic molecules [11], as well as the formation of protein-DNA and Cr-DNA adducts, DNA strand breaks, and DNA–DNA cross-links [12]. 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 [8] or were hypothesized to occur in yeast [13]. However, the detailed mechanisms regarding yeast-Cr interactions are yet unclear.

In this study, Cr(VI) tolerance in yeasts isolated from wastewater effluents was investigated. Chromate reductases (ChRs) involved in Cr(VI) reduction in yeast cells were characterized. Proteins extracted from yeasts were further explored for one-dimensional (1DE) gel electrophoresis. The potential of yeasts for bioremediation of chromium was also studied.

2. MATERIALS AND METHODS

Isolation and screening. Wastewater samples were collected from industrial effluents of Shiekhupura located near Lahore and their physiochemical parameters viz., pH, and temperature were noted. Cultures were maintained on YPD agar plates comprised of glucose 20 g/dm^3, peptone 20 g/dm^3, yeast extract 10 g/dm^3, and agar 20 g/dm^3. Isolation was done by spreading 100 µl of wastewater samples on YPD agar plates. For screening, yeast isolates were aerobically grown in salt medium containing: 10 g/dm^3

of glucose, 1 g/dm^3 of $(NH_4)_2SO_4$, 0.15 g/dm^3 of KH_2PO_4, 0.1 g/dm^3 of K_2HPO_4, 0.1 g/dm^3 of $MgSO_4 \cdot 7H_2O$, 0.026 g/dm^3 of $FeSO_4$ and 0.086 g/dm^3 of $CaCl_2$. pH of the medium was adjusted to 7.0–7.2; the media was sterilized at 121 °C for 20 min, inoculated with yeast cultures. The isolated strains were evaluated for their tolerance to Cr(VI) ions (50–250 mg/dm^3) and the highest resistant strains were selected for further experiments. Controls were treated identically but without heavy metal exposure.

Survival to heavy metal exposure. Minimum inhibitory concentration was assessed by growing cultures on YPD agar plates with increasing concentrations of Cr(VI) ions ranging from 50 to 500 mg/dm^3 [14]. Grown yeast cells were subsequently transferred at a given concentration to the next concentration and maximum resistance was evaluated until *P. kudriavzevii* AM-4 and *Candida* sp. PSM-33 cells were unable to grow as colonies on metal-containing agar plates. Any color change of cultures in response to metal exposure was carefully visualized. The growth curves were determined by growing the yeast isolates in minimal salt medium (MSM) broth in the presence and absence of 150 mg/dm^3 of Cr(VI) at 30 °C and pH of 7.0. The growth was monitored by taking optical density (OD) at 600 nm after a regular interval of 4 h up to 36 h.

Intracellular enzyme activity. Intracellular chromate reductase enzyme activity was estimated by collecting the cell pellet after centrifugation (1500*g* for 10 min) of culture, washing twice with sodium phosphate buffer (50 mM, pH 7.0) and lysing by sonication for 15 s with a 60 s interval (5 cycles) at 4 °C. This sonicated suspension or cell-free extract (CFE) was collected by centrifugation (4000*g*) for 10 min and used as a crude enzyme source for activity analysis. The protein concentration was determined by the Bradford [15] assay using bovine serum albumin (BSA) as a standard.

Chromate reductase (ChR) activity was determined utilizing an enzyme assay comprised of the crude enzyme, 0.1 mM NADH, and 1 mg/dm^3 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 [16] and the percentage relative activity of the crude enzyme was calculated by the change in absorbance in the reaction mixture. 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 under the assay conditions at 30 °C.

Reduction of hexavalent chromium. The reduction was estimated by measuring the decrease in hexavalent chromium in the culture filtrate by the diphenylcarbazide (DPC) method [17]. The reaction mixture was kept at room temperature for 10 min for pink-violet colored complex formation in acidic solution and optical density (OD) 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 the crude enzyme was determined by incubating the 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 OD Chloride metal salts such as NaCl, $MgCl_2$, $CoCl_2$, $HgCl_2$, and $CaCl_2$ were selected to assess their effects on enzyme activity. The concentration of 5 mg/dm^3 of each metal ion was used. A reaction mixture with no metal ion added in the reaction was taken as control.

Chromium uptake process by yeasts and reductase induction. Cultures were grown in MSM medium, incubated under shaking condition (120 rpm) and aliquots (5 cm^3) were taken out under sterilized conditions after time intervals of 2, 4, 6, 8, 10, and 12 days. The samples were centrifuged at 4000*g* for 10 min. Collected culture pellets were weighed and washed thrice with autoclaved distilled water, afterward 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 a soluble fraction. The second part treated with 0.2 M HNO_3 (1:1) for acid digestion was left on a hot plate for half an hour. Total chromium content present in the medium or processed by the yeast cells was estimated utilizing an atomic absorption spectrometer (Zeeman AAS, Z-5000 Model, Hitachi Ltd., Japan) with an air-acetylene burner at 359.3 nm [18, 19].

One dimensional gel electrophoresis was performed on 14% a polyacrylamide gel. Protein samples (20 μg/dm^3) 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 of 120 V according to Laemmli [20]. The relative position of protein bands was determined with a protein marker.

Statistical analyses. Three independent experiments were performed and average values of means±standard deviation (*SD*) data have been presented. A control group has not been exposed to any treatment. Student's *t*-test and the analyses were done with the program statistical package for social sciences (SPSS) version 15. The resulting values were expressed as mean±*SD* where $^{*}p = 0.05$ and $^{**}p = 0.1$.

3. RESULTS

Both yeast isolates, *Candida* sp. PSM-33 previously isolated by Ilyas et al. [21] with accession number KJ913821 and *P. kudriavzevii* AM-4 previously reported by Ilyas and Rehman [22] with accession number JN009854, showed maximum resistance to Cr(VI)

up to 350 and 400 mg/dm^3, respectively. Biomass content in both yeasts was decreased as compared to the controls. The color of both yeast isolates was affected after adding Cr(VI) and transformed in comparison with the control which was probably a stress response imposed by yeasts to chromium (Fig. 1). This color change may be due to yeast cells adapt and synthesis essential enzymes required for the accumulation and reduction of Cr(VI) to Cr(III).

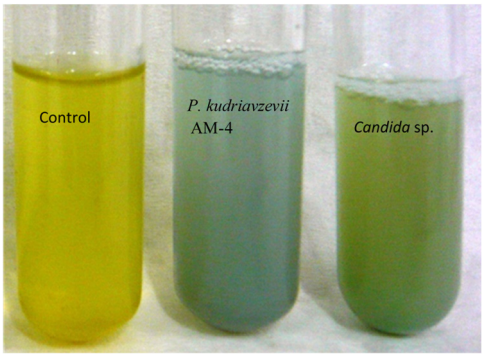


Fig. 1. The ability of *Candida* sp. PSM-33 and *P. kudriavzevii* AM-4 to reduce hexavalent chromium (50 mg/dm^3) by color change grown in a minimal salt medium after 48 h of incubation

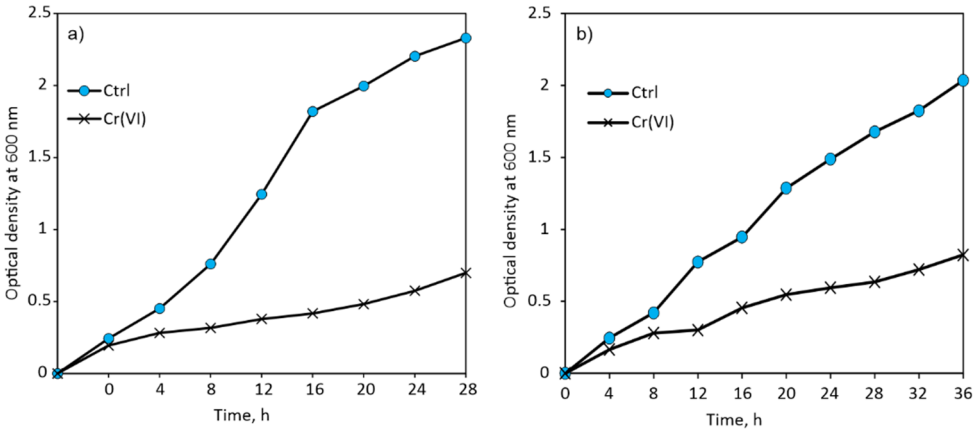


Fig. 2. Growth curves of *Candida* sp. PSM-33 (a) and *P. kudriavzevii* AM-4 (b) with Cr(VI) (150 mg/dm^3) and without Cr(VI) after 36 h of incubation

The slow growth patterns of *Candida* sp. PSM-33 and *P. kudriavzevii* AM-4 strains were determined under 150 mg/dm^3 Cr(VI) stress. The slower growth in the presence of Cr was due to a low proliferation rate. Growth rates (Figs. 2a, b) of both yeast isolates were extended in the presence of Cr(VI) as compared to the controls. Both the strains raised and induced the intracellular chromate reductase (ChR) activity by reducing Cr(VI) present in the culture medium. *P. kudriavzevii* AM-4 exhibited 1.8-fold whereas *Candida* sp. PSM-33 0.3-fold increase in ChR activity when compared to controls (Fig. 3).

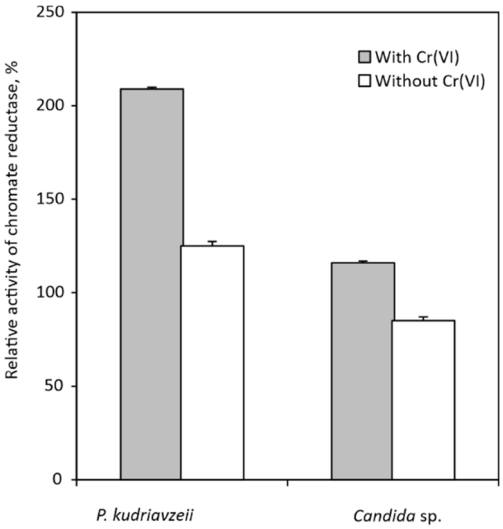
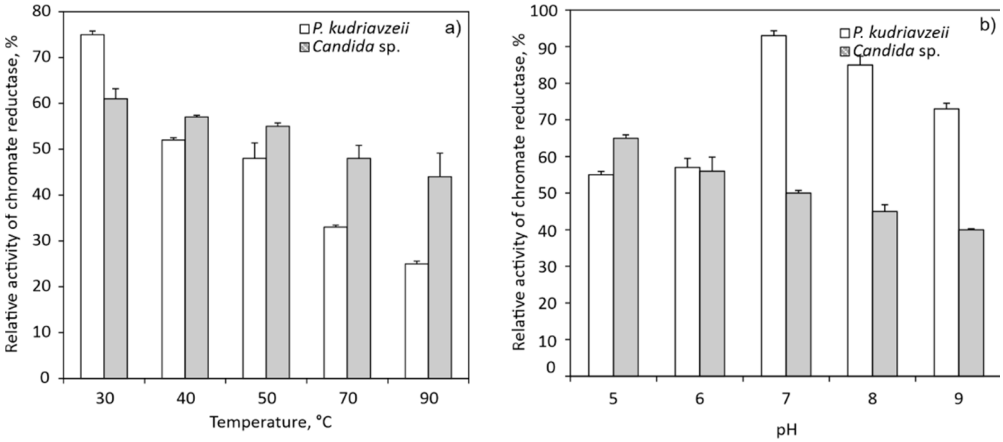


Fig. 3. Relative specific activity of chromate reductase in *Candida* sp. PSM-33 and *P. kudriavzevii* AM-4



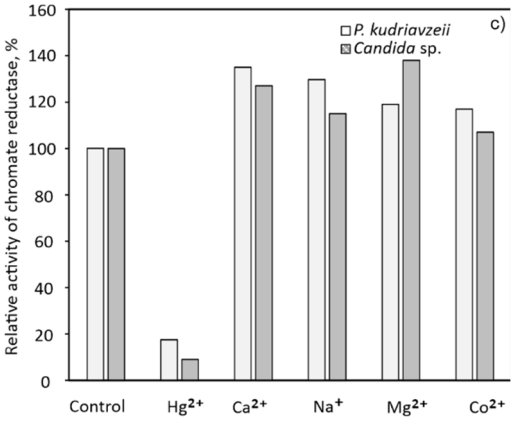


Fig. 4. Effect of temperature (a), pH (5–9) (b), and metal ions (5 mg/dm^3) (c) on chromate reductase activity pattern of *Candida* sp. PSM-33 and *P. kudriavzevii* AM-4

Chromate reductases (ChRs) from both yeasts isolates exhibited maximum activity at 30 °C (Fig. 4a). pH is a major factor affecting the efficiency of enzymes as acidic or strongly alkaline pH inactivates enzymes. Chromate reductase was moderately stable at pH ranging from 5 to 6 in *P. kudriavzevii*. Its optimum pH was 7 in sodium phosphate buffer (Fig. 4b). Chromate reductase activity decreased to 38% in *P. kudriavzevii* AM-4 with decreasing pH and to 25% in *Candida* sp. PSM-33 with increasing pH. At pH above or below the optimum, a decrease in enzyme activity was pronounced.

The presence of heavy metal ions in industrial effluents could change chromate reductase activity. The enzyme activity was also sensitive to metal ions. Ca^{2+} and Na^{+} ions enhanced enzyme activity to 35 and 30% while Mg^{2+}, and Co^{2+} ions activated the enzyme more modestly (Fig. 4c) in *P. kudriavzevii* AM-4. Mg^{2+} and Ca^{2+} enhanced chromate reductase in *Candida* sp. PSM-33 to 27 and 38%, respectively. *Candida* sp. PSM-33 showed a negligible increase in enzyme activity after Co^{2+} addition. Relative chromate reductase activity was highest when treated with Ca^{2+}, Na^{+}, and Mg^{2+} but other divalent metal ions also increased enzyme activity suggesting that it is not specific for cations. Inhibition of enzyme activity by Hg^{2+} was noticed which was higher (91%) in *Candida* sp. PSM-33 and reduced to (83%) in *P. kudriavzevii* AM-4 (Fig. 4c).

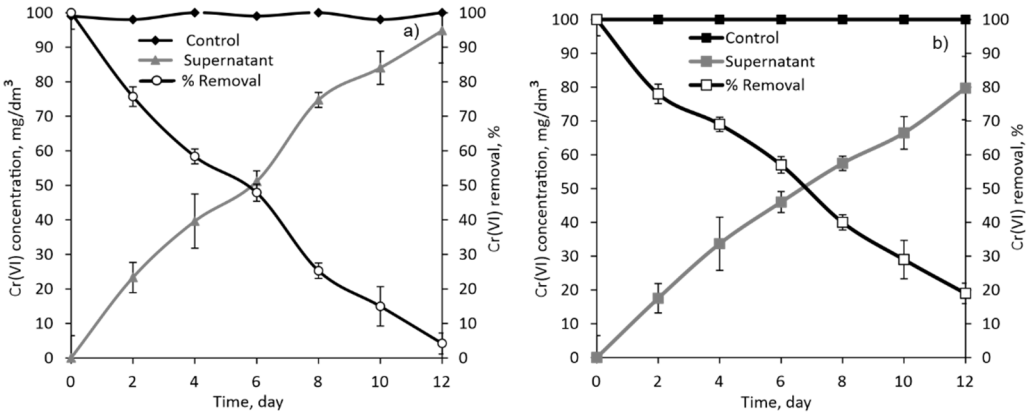


Fig. 5. Time dependences of estimated concentration of chromium from the culture supernatants of *Candida* sp. PSM-33 and *P. kudriavzevii* AM-4

Supernatants of *P. kudriavzevii* AM-4 exhibited 76, 60, 49, 26, 17, 5 mg/dm^3 of chromium after 2, 4, 6, 8, 10, and 12 days and the removal potential was 23–94% (Fig. 5a). The highest uptake potential in *P. kudriavzevii* AM-4 was 17, 29, 37, 58, 65, and 73 mg/g and the cells adsorbed 6, 9, 13, 16, 18, and 21 mg/g (Fig. 6a). *Candida* sp. PSM-33 culture supernatants showed 78, 69, 57, 35, 20, and 11 mg/dm^3, and the chromium removal efficiency ranged between 21 and 88% (Fig. 5b) within 2 and 12 days. The maximum chromium uptake potential determined in *Candida* sp. PSM-33 was 16, 23, 30, 48, 61, and 68 mg/g and the cells could adsorb 5, 8, 12, 15, 18, and 20 mg/g on

the cell walls (Fig. 6b) after 12 days. These results indicate that both yeasts were highly capable of accumulating chromium although the removal efficiency in *P. kudriavzevii* AM-4 was higher than that of *Candida* sp. PSM-33.

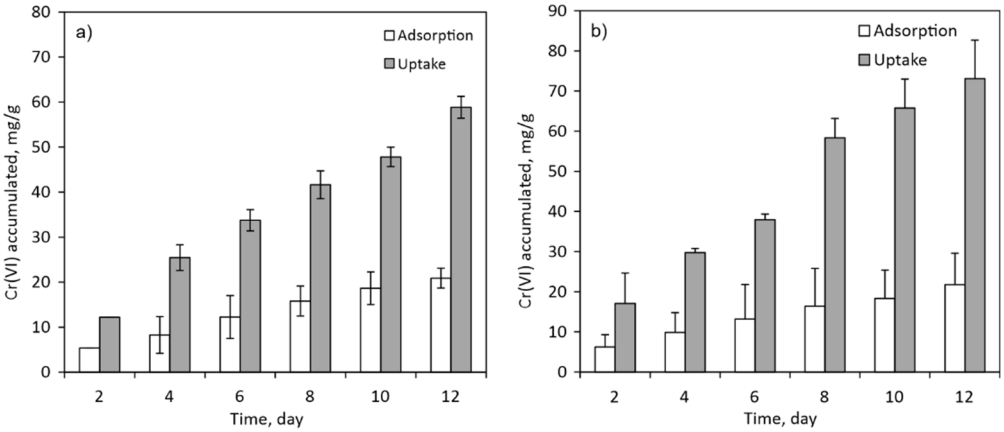


Fig. 6. Time dependences of uptake and adsorption of Cr(VI) by *Candida* sp. PSM-33 (a) and *P. kudriavzevii* AM-4 (b)

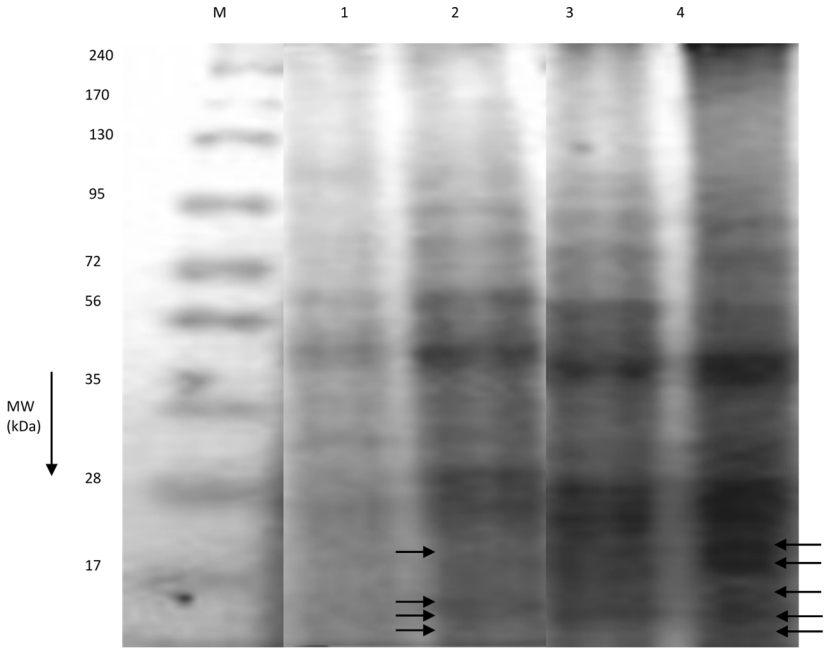


Fig. 7. Results of SDS-PAGE analysis of total proteins extracted from *Candida* sp. PSM-33 and *P. kudriavzevii* AM-4 stained with Coomassie blue R-250. Lane M, protein maker; lanes 1 and 3 show control, whereas lanes 2 and 4 illustrate Cr treated

Expression of proteins in metal treated and untreated cultures were explored by one--dimensional gel electrophoresis. Known protein concentration (20 μg/dm^3) was used and most of the differentially expressed protein bands were seen in yeast cultures exposed to chromium. Chromium treated *Candida* sp. PSM-33 showed increased intensity of 20 kDa protein band which was much weaker in control and some additional proteins, i.e., 14, 12, and 8 kDa were also induced under Cr-stress condition (Fig. 7). Likewise, in *P. kudriavzevii* AM-4, stronger 22, 20, and 10 kDa relative protein bands were present in Cr treated samples while 15 and 8 kDa were induced by Cr (Fig. 7). Protein banding patterns revealed different intensities between control and treatments although overall protein loadings were equivalent.

4. DISCUSSION

Microbes utilize different strategies for their survival under metal stress; consume trace amounts of metal ions for their metabolism; resist and/or detoxify excessive toxic amounts of heavy metal present. Thus, microorganisms have the potential to uptake metal ions through the processes of bioaccumulation, biosorption, and the efflux system. Chromate reduction has been reported mostly in bacteria; however, few cases have been reported for yeasts, fungi, and actinomycetes [23].

In the current investigation, both *Candida* sp. PSM-33 and *P. kudriavzevii* AM-4 resisted up to 350 and 400 mg/dm^3 of hexavalent chromium. Recently, Elahi and Rehman [24] reported that *Trichosporon asahii* and *Rhodotorula mucilaginosa* can tolerate Cr(VI) up to 400 and 300 mg/dm^3, respectively, showing good agreement with our results. *Trichosporon asahii* can tolerate $NaAsO_2$ and $CdCl_2$ up to 3900 mg/dm^3 and 1100 mg/dm^3, respectively [14]. Li et al. [18] stated that *Rhodotorula* sp. Y11 isolated from mine soil could survive under cadmium concentration of 2 g/dm^3.

Candida intermedia showed an extended lag growth phase at 50 mg/dm^3. Ilyas et al. [21] also reported an extended lag phase of *C. tropicalis* cultures grown in a medium containing heavy metal ions. In this study, the growth was slowed down in the presence of metal ions as compared to the control cultures containing no metal ions (Fig. 2).

Das and Chandra [25] noticed an increase in chromate reductase activity when working with Cr(VI) *Streptomyces* sp. M3 cultures. The enzymatic bio-speciation of Cr(VI) to Cr(III) with eukaryotic microorganisms was reported in *Candida maltose*, *Candida utilis* and fungi such as *Hypocrea tawa* and *Aspergillus* sp. [26]. Microbes of the genera *Pseudomonas*, *Arthrobacter*, *Escherichia* and *Bacillus* have been reported to reduce Cr(VI) through soluble chromate reductase [27]. The negligible reduction was observed in controls (Fig. 1) indicating the role of yeast's enzymes in Cr(VI) reduction.

Maximum chromate reductase activity observed by *Pichia jadinii* M9 and *Pichia anomala* M10 was 60 and 50 °C, respectively [28]. Among bacterial ChRs, the optimal temperature ranges from 30 to 50 °C [27, 29]. The ChR of *Candida* sp. PSM-33 had

higher activity in sodium acetate buffer at pH 5 in contrast with the reports of Martorell et al. [28]. In the current study, the optimum temperature of ChR in both yeast strains was 30 °C while *Candida* sp. PSM-33 showed the higher ChR activity at pH 5 whereas *P. kudriavzevii* AM-4 exhibited maximum activity at pH 7. ChR activity was increased in the presence of Mg, Ca, Co, and Na but was inhibited in the presence of Hg cations. These results agree with reports of Camargo et al. [30] and Elangovan et al. [27].

Li et al. [18] reported a maximal metal uptake of 19.38 mg/g by *Rhodotorula* sp. Y11. The highest chromium adsorption by *Candida* sp. PSM-33 and *P. kudriavzevii* AM-4 indicated more binding sites on cell walls as well as their potential use as biosorbent and effectiveness to remove Cr(VI) ions from the wastewater. Yeasts can accumulate a higher concentration of heavy metal ions by the 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 [12].

Heavy metals (Cd, Cu, Pb) uptake by a consortium of yeasts, Penicillium sp. A1 and *Fusarium* sp. A19 was significantly higher as revealed by Pan et al. [31]. The maximum biosorption capacity of macro fungus, *Amanita rubescens* determined was 27.3 mg/g for Cd. Engineered *Saccharomyces cerevisiae* cells have been shown enhanced arsenite accumulation by overexpression of transporters responsible for the metal influx. Biosorption and uptake are mainly used to treat water containing a 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 environmentally 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 [12, 18, 28].

In the present study, low molecular-weight protein bands were abundant in all chromium treated samples (Fig. 7) and they may correspond to metallothionein proteins (MTs) which play a significant role in the protection and survival of microorganisms including yeasts against metal oxidative stress. Certain protein bands appearing in the 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. The overexpression and disappearance of the protein band in the presence of metal ions have also been reported by Durve et al. [32]. Ilyas and Rehman [22] reported some protein bands in the mass range of <14 kDa in metal treated *P. kudriavzevii* AM-4 and *T. asahii* samples. Similarly, Elahi and Rehman [24] found 16 and 20 kDa protein bands with much higher intensity in Cr-treated *R. mucilaginosa* samples as compared to the control.

5. CONCLUSIONS

Sewage yeast isolates *Candida* sp. PSM-33 and *P. kudriavzevii* AM-4 showed significant resistance towards chromium. Minimum inhibitory concentration was assessed and the growth pattern was slower in Cr-treated cultures as compared to the control. The

crude enzyme exhibited maximum activity at 30 °C (pH 5) in *Candida* sp. PSM-33 and 30 °C (pH 7) in *P. kudriavzevii* AM-4. The enzyme activity pattern was not affected to a higher extent in the presence of metal ions. Cr(VI) was absorbed and adsorbed by the yeast cells and reduced to Cr(III) by chromate reductase. Certain bands present in controls were absent in treatments and the production of low molecular-weight metallothionein proteins (MTs) was abundant in metal-induced samples. The current investigation demonstrated that both yeast strains have promising potential to reduce toxic and soluble Cr(VI) to less toxic and insoluble Cr(III) and hence can be employed as a bio-sorbent for Cr(VI) detoxification from the contaminated effluents.

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