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Research Article

Heavy metal tolerance in the psychrotolerant *Cryptococcus* sp. isolated from deep-sea sediments of the Central Indian Basin

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Abstract

A deep-sea isolate of the psychrotolerant yeast *Cryptococcus* sp. (NIOCC#PY13) obtained from polymetallic nodule-bearing sediments of the Central Indian Basin was examined for its capacity to grow in the presence of various concentrations of the heavy metal salts i.e., $ZnSO_4$, $CuSO_4$, $Pb(CH_3COO)_2$ and $CdCl_2$. It demonstrated considerable growth in the presence of 100 mg/l concentrations of the above-mentioned four heavy metal salts both at 30°C and 15°C. This strain tolerated comparatively higher levels of these four metal salts than other deep-sea and terrestrial yeast isolates belonging to *Cryptococcus*, *Rhodotorula*, *Rhodosporidium* and *Sporidiobolus* spp. Optimum pH for growth of this isolate was in the range of 6–8 in the presence of heavy metal salts at these two temperatures. Scanning electron microscopic (SEM) studies exhibited altered cell surface morphology of the cells under the influence of heavy metals compared to that with control. The adsorption of heavy metals to the cells was demonstrated by FTIR and EDAX analysis. As evidenced by atomic absorption spectrophotometric (AAS) analysis, about 30–90% of the heavy metals were removed from the culture supernatant after 4 days of growth at 30°C. This deep-sea yeast isolate appears to be a potential candidate for bioremediation of metal-contaminated sites. Moreover, its metal tolerance properties provide a significant insight into its ecological role and adaptations to growth in such extreme conditions. Copyright © 2013 John Wiley & Sons, Ltd.

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Keywords: **heavy metals; biosorption; metal tolerance; deep-sea sediments; Central Indian Basin; *Cryptococcus* sp.**

Introduction

The presence of large amount of metals in the sediments of deep-sea hydrothermal vents is a major feature (Malahoff, 1985). However, very little is known about the presence of heavy metals in the non-vent sediments of the deep sea. A few studies have reported some of the heavy metals, such as Cr, Pb, Zn and Co, to be present in the deep-sea sediments also (Schnetger *et al*., 2000). Nath *et al*. (1989, 2005) reported the presence of various major and minor elements, including Pb, Zn, Cu and Co, in deep-sea sediments of the Central Indian Basin. Resistance to heavy metals in heterotrophic bacteria from hydrothermal vent environment is reported by several workers (Jeanthon and Prieur, 1990a, 1990b). Kato *et al*. (1996), Abe and Horikoshi (2001) and Abe *et al*. (2004) have reported potential biotechnological applications of piezophilic bacteria from deep-sea sediments. Metal-resistant bacteria have also been isolated and characterized from sediment of deep-sea Mn nodules of the Pacific Ocean (Tian and Shao, 2006). A Mn(II)-oxidizing deep-sea bacterium was demonstrated to have potential

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application in multiple heavy-metal removing systems (Wang *et al.*, 2009). One of the yeast strains, N6 belonging to *Cryptococcus liquefaciens* (Abe *et al.*, 2006), isolated from deep-sea sediments of the Japan Trench was demonstrated to tolerate very high concentrations of Cu, which was triggered by production of an antioxidant enzyme, superoxide dismutase, as a defensive mechanism (Abe *et al.*, 2001). The greater capacity of deep-sea isolates towards metal tolerance abilities may be attributed to their ability to survive under extreme conditions by expression of genes involved in combating these stress mechanisms (Abe and Minegishi, 2008; Singh *et al.*, 2012). Since the exposure to heavy metals also imposes similar stress conditions in microorganisms, deep-sea microbes for the metal tolerance studies may prove highly effective for the bioremediation of metal-contaminated sites.

Diverse fungal and yeast isolates have been reported from deep-sea sediments of the Central Indian Basin (Damare *et al.*, 2006; Singh *et al.*, 2010). These fungal and yeast isolates exhibited growth under low-temperature and elevated hydrostatic pressure conditions (Singh *et al.*, 2010). One of the yeasts, NIOCC#PY13, identified as *Cryptococcus* sp. by amplification and sequencing of the *18S* SSU-rDNA region was psychrotolerant, exhibiting growth at 15°C (Singh *et al.*, 2010). The present study is the first report demonstrating greater tolerance and biosorption characteristics of this deep-sea *Cryptococcus* sp. towards different concentrations of four heavy metal salts. This study opens new avenues to explore the deep-sea organisms for bioremediation capabilities and also provides information regarding their possible ecological role under such extreme conditions.

Materials and methods

Yeast strain and culture conditions

The psychrotolerant yeast NIOCC#PY13 (*Cryptococcus vishniacii)* was isolated from deep-sea sediment of the Central Indian Basin (10–16.5°S, 72–77°E) on board the Russian research vessel *Akademic Boris Petrov* during Cruise No. ABP26 in December 2006. The isolate was maintained at 5°C on malt extract agar medium by subculturing at an interval of 20–25 days. It was identified by amplification and sequence analysis of the partial *18S* region of SSU-rDNA. The *18S* sequence was submitted to the GenBank NCBI database under Accession No. EU723510. The culture showed growth at both 15°C and 30°C (Singh *et al.*, 2010).

Effect of different heavy metals on the growth pattern of NIOCC#PY13

The yeast isolate was grown in 20 ml YPD medium (0.5% yeast extract, 1% peptone and 1% dextrose) and incubated overnight at 30°C on a rotary shaker incubator at 170 rpm for preparation of the inoculum; 1 ml of this culture was further inoculated into 100 ml YPD medium containing 0 (control), 10, 50 and 100 mg/l concentrations of four heavy metals, $ZnSO_4$, $CuSO_4$, $Pb(CH_3COO)_2$ and $CdCl_2$. The respective mM concentrations of these four heavy metal salts are represented in Table 1. The flasks containing the media were incubated at 30°C and 15°C on a rotary shaker at 170 rpm. The growth was monitored at intervals of 24 h by taking the absorbance at 600 nm. Controls were incubated with four concentrations of the above metal salts in the YPD medium without any culture inoculum to monitor the precipitation of these salts, and the readings were subtracted from respective media flasks containing the yeast culture. All the experiments were performed in triplicate and the results are represented by average values.

Minimum inhibitory concentration (MIC) values were determined by streaking the isolated colonies of yeast on YPD agar medium plates containing 10 mg/l concentrations of $ZnSO_4$, $CuSO_4$, $Pb(CH_3COO)_2$ and $CdCl_2$; the plates were incubated at 30°C for 48 h. This process was repeated with successively higher concentrations of the above four metal salts until the MIC of each metal was obtained. The MIC is defined as the lowest concentration of the

Table 1. Concentrations of the four heavy metal salts used in the present study (mg l^{-1} and corresponding mM values)

	Concentration					
Metal salt	**mg l^{-1}**	**mM**	**mg l^{-1}**	**mM**	**mg l^{-1}**	**mM**
$ZnSO_4$	10	0.062	50	0.31	100	0.62
$CuSO_4$	10	0.062	50	0.31	100	0.62
$Pb(CH_3COO)_2$	10	0.03	50	0.15	100	0.31
$CdCl_2$	10	0.05	50	0.27	100	0.54

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heavy metal added to a media plate at which the yeast isolate streaked from a single colony did not grow.

Effect of pH on the growth of NIOCC#PY13 in the presence of heavy metals

To analyse the effect of pH on growth of yeast in the presence of the above four heavy metals, the inoculum was prepared in a similar way to that described above. A range of pH values were selected (2, 4, 6, 8 and 10) for the experiment. The pH of the growth medium was adjusted to the required values using 0.1 N HCl or 0.1 N NaOH. Inoculum (1 ml) was added to 100 ml YPD medium at different pH values containing 100 mg/l concentrations of the four heavy metals, $ZnSO_4$, $CuSO_4$, $Pb(CH_3COO)_2$ and $CdCl_2$. The growth was measured by taking the absorbance at 600 nm after 4 and 7 days of incubation at 30°C and 15°C, respectively. Controls for each pH value were incubated to monitor the precipitation of these metal salts, as described in the previous section.

Effect of heavy metals on the cell morphology

The yeast isolate was grown in the presence of 100 mg/l concentrations of the heavy metals at 30°C for 3 days. The cell suspension was fixed on small glass pieces and dehydrated sequentially with a series of ethanol solutions (10%, 20%, 30%, 50%, 70% and 90%), prepared with distilled water. The samples were coated with a thin layer of gold using a sputter-coater (SPI-MODULE Gold Sputter Coater) to increase the electron conduction and to improve the quality of the micrographs. The cell morphology was observed with a scanning electron microscope (SEM; Jeol Model 5800 L, Japan). The acceleration voltage was constant at 20 kV and the microprobe was focused at magnifications of ×190, ×230 and ×250.

Analysis of heavy metals sorption to yeast cell surface

Energy-dispersive X-ray analysis (EDAX) and Fourier transform infrared spectroscopy (FTIR) were employed to examine the sorption of the four heavy metals on the cell biomass of this isolate. It was grown in the presence of 100 mg/l concentrations of the heavy metals at 30°C for a period of 3 days. The cell suspension was centrifuged at 8000 rpm for 10 min at 4°C. The cell pellet was lyophilized for 24 h and the dried biomass was subjected to EDAX and FTIR analysis. EDAX was performed in a similar manner to SEM, as described in the previous section.

Fourier transform infrared spectroscopy (FTIR) spectra were recorded in the range 500–4000 cm^{-1}, using an FTIR (Model 8201PC, Shimadzu, Japan) with 1 cm^{-1} resolution. Pellets were prepared by mixing the lyophilized samples with 50 mg KBr, using a diffused reflectance spectroscopy accessory. The FTIR spectra of lyophilized biomass before and after sorption were recorded.

Analysis of residual heavy metal ions in the culture supernatant

The yeast isolate was grown in the presence of three different concentrations of the heavy metal salts (10, 100 and 200 mg/l) for 3 days. The concentration of residual heavy metal ions (Cu, Pb and Zn) in the supernatant was determined using a flame atomic absorption spectrophotometer (Thermo Electron Corp., S-Series, SOLAR S AAS) with an air–acetylene flame at specific wavelengths for all of the above elements. Blanks and standards were run in a similar manner for calibration. Deuterium background correction was used. The instrument was calibrated by running blank and standard solutions prior to each element analysis. A recalibration check was performed at regular intervals after every 10 readings. All chemicals used in the study were of analytical grade. As standards with comparable matrices were not available, a certified reference standard from the US Geological Survey (Nod A-1: Nodule standard) was digested and run to test the analytical and instrument accuracy during the time of the analyses. The concentration of residual Cd metal ions could not be estimated, due to non-availability of the Cd lamp for the above spectrophotometer.

Results and discussion

Effect of temperature and pH on metal tolerance properties of NIOCC#PY13

The yeast isolate used in the present study exhibited considerable growth in the presence of all the three concentrations (10, 50 and 100 mg/l) of the four heavy metals, Pb, Cu, Zn and Cd. Growth in the

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presence of 100 mg/l heavy metals was comparable to the control at both 30°C and 15°C (Figure 1a, b). However, at 15°C the time taken to reach the stationary phase was longer than at 30°C. The order of toxicity was found to be Cd > Cu > Zn > Pb for growth at these two temperatures. A similar observation has been reported where yeast strains were found to tolerate higher concentration of Zn than Cu (Vadkertiova and Slavikova, 2006). However, the level of Cu tolerated by the deep-sea yeast in the present study was comparatively lower than the results obtained by Abe *et al.* (2001), where a deep-sea *Cryptococcus* sp. could grow on solid medium containing 50 mM $CuSO_4$. The MICs of the four heavy metals used in the present study were in the range 900–1400 mg/l. These results demonstrated a high level of heavy metal tolerance of this deep-sea yeast isolate at both 30°C and 15°C.

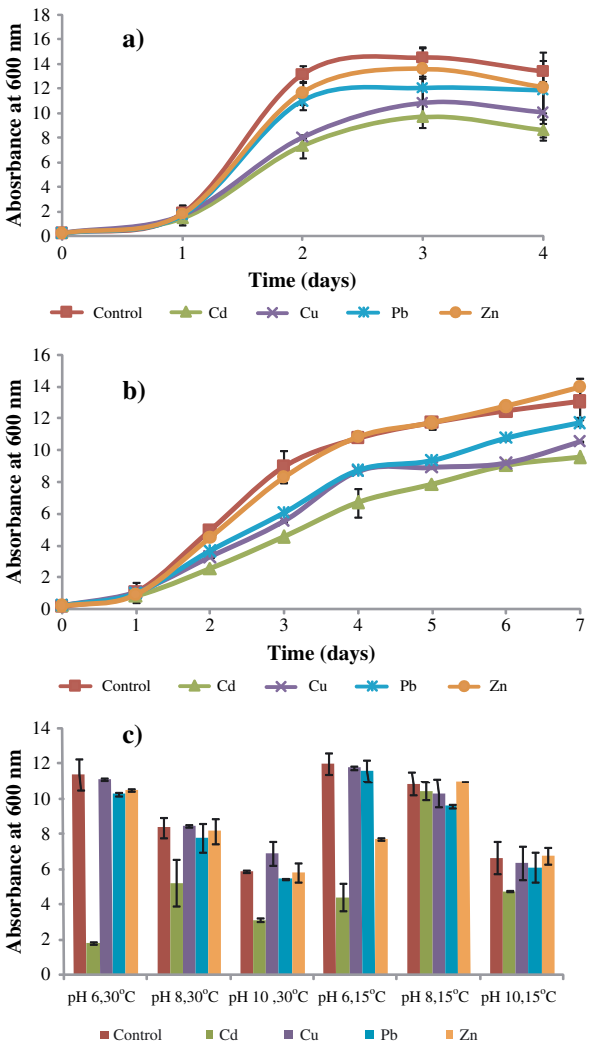

Figure 1. The growth pattern of the deep-sea yeast isolate NIOCC#PY13 in the presence of 100 mg/l concentrations of four heavy metals at: (a) 30°C; (b) 15°C; and (c) different pH and temperature conditions (A_{600} measurements were taken on days 4 and 7 for 30°C and 15°C, respectively)

Optimum pH for the growth of the yeast isolate was found to vary for different metals and temperatures (Figure 1c). The isolate showed good growth with 100 mg/l concentrations of Zn, Pb and Cu at a pH range of 6–8, whereas for Cd, pH 8 was found to be the most favourable (Figure 1c). A combination of low temperature (15°C) and pH 8 enhanced the growth of the yeast in the presence of Cd, almost reaching the value of the control. In general, optimum pH for metal uptake has been reported to be between 4 and 8 for a wide range of microbes (Fourest and Roux, 1992; Brady and Duncan, 1994). Previous studies have reported pH to be a significant factor for the biosorption of heavy metals to microbial biomass, influencing the solution chemistry of the heavy metals (Özer and Özer, 2003; Tewari *et al.*, 2005). With increasing pH, relatively more ligands would be exposed carrying negative charges, resulting in the subsequent attraction of metal cations and biosorption onto the binding sites on the cell surface (Brady and Duncan, 1994; Delgado *et al.*, 1998). The biosorption capacity of *Mucor rouxii* cells for Pb(II) was found to increase with increasing pH value and reached a maximum at pH 5 (Yan and Viraraghavan, 2003).

Similarly, the yeast isolate used in the present study showed better growth in the presence of different metals with increasing pH, with the best results at pH 6 for Pb, Zn and Cu at both 30°C and 15°C (Figure 1c). This suggests that at this pH the cell surface ligands of the yeast *Cryptococcus* sp. responsible for binding of these three metal cations in the solution are most efficiently exposed. In contrast, pH 8 at both temperatures was found to be suitable for better growth of this yeast in the presence of Cd (Figure 1c). The composition of extracellular glycoproteins has been reported to influence the adsorption and tolerance level of Cd by different yeast species (Breierová *et al.*, 2002). A similar phenomenon may be suggested for enhanced tolerance towards Cd at low temperature and pH 8 in the present study by alteration of extracellular glycoproteins responsible for Cd uptake on yeast cell surface.

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Effect of heavy metals on cell morphology

SEM micrographs of the yeast indicated clear changes in cell surface morphology after growth in the presence of the heavy metals (Figure 2). The clear damage to yeast (Figure 2a) could be observed by the presence of shrunken and distorted cell walls in the presence of Cd (Figure 2b) and depressions in the presence of Pb and Zn (Figure 2d, e). Least visible damage was noticed with Cu-loaded yeast cells (Figure 2c). Although the cell morphology was affected in the presence of the heavy metals, their overall growth was not much influenced. Various factors may be responsible for such alterations in cell surface morphology of microbial biomass in the presence of heavy metals. Secretion of extracellular polymeric substance by *Desulfovibrio desulfuricans* during biosorption of Zn and Cu was reported to modify its cell surface morphology (Chen *et al*., 2000). Biosorption of Zn, Ni and Cr was demonstrated by SEM analysis using fungal biomass of *Aspergillus* sp. (Kumar *et al*., 2012).

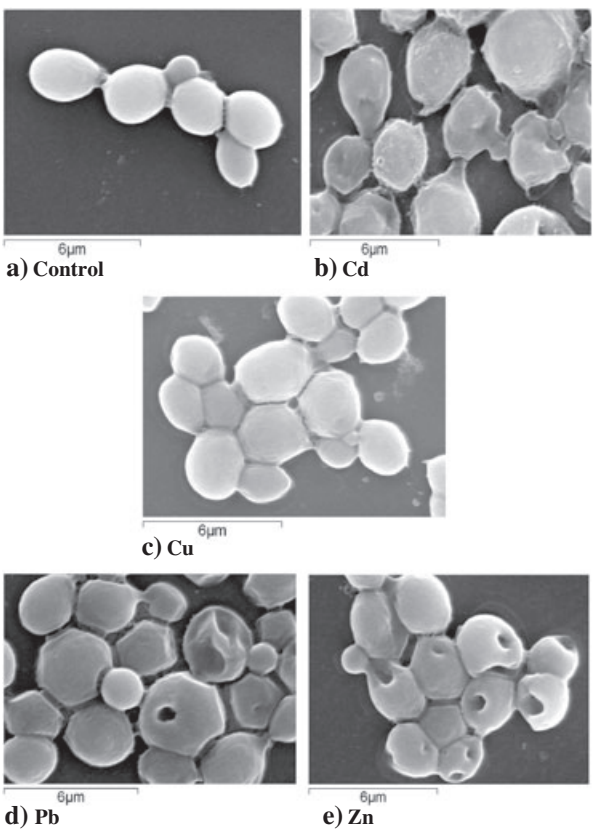

Figure 2. SEM images of NIOCC#PY13 grown in the presence of different heavy metals at a concentration of 100 mg/l

EDAX analysis for biosorption of heavy metals

The EDAX spectra of the deep-sea yeast grown in the presence of three different heavy metals (Cd, Cu and Pb) showed the presence of corresponding metal peaks (Figure 3), demonstrating their biosorption on cell surface. No positive result was observed for EDAX spectra of the yeast cells grown in the presence of Zn. One possible explanation for this may be the very different texture of yeast biomass after growth in the presence of Zn. The yeast cells turned into powdered form when dried after growth with Zn in the medium and did not stick to the sputter, causing undetected peaks in the EDAX spectra. EDAX spectra have been used successfully to demonstrate metal biosorption by microbial biomass (Raize *et al*., 2004; Cabuk *et al*., 2007). However, a much more detailed study involving metabolic phases of the yeast is recommended in future, which could provide a clearer picture of the metal uptake mechanism.

FTIR analysis of the yeast biomass

FTIR spectra of the pure biomass of yeast isolate was compared with the biomass loaded with 100 mg/l concentrations of the four heavy metals to examine the functional groups' changes due to metal ion interactions (Figure 4). Several new peaks appeared in the spectral pattern of metal-loaded biomass compared with the control. Spectral changes in Pb- and Zn-loaded biomass were prominent in the region 800–1700 cm^{-1}. However, Cd- and Cu-loaded biomass exhibited comparatively fewer peak shifts than the control biomass (Figure 4a–c). The spectral changes in the region 1600–1700 cm^{-1} were observed in all four heavy metal-loaded biomasses of the yeast isolate (Figure 4a–e). The peak range 1500–1700 cm^{-1} is the stretching vibration band of C=O of the amide bond, suggesting stretching of this functional group as a result of metal biosorption. Similar results were reported by Çabuk *et al*. (2007), where amide groups were found to be involved in biosorption of Pb(II) ions by indicating a peak shift at 1633 cm^{-1} in the FTIR spectrum of Pb-loaded *S. cerevisiae* biomass.

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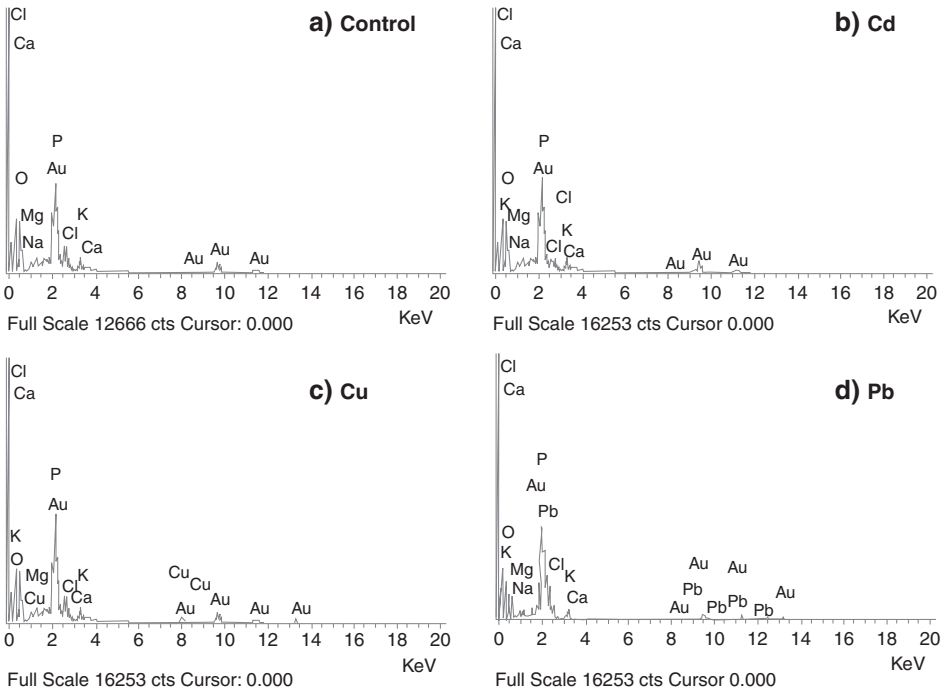

Figure 3. EDAX spectra of NIOCC#PY13, representing biosorption of different heavy metals: (a) control; (b) Cd; (c) Pb; and (d) Cu. All of these heavy metals were used at a final concentration of 100 mg/l. EDAX spectra of NIOCC#PY13 in the presence of Zn did not show any significant peaks and therefore is not represented in the figure

The new peaks appeared in the 1500–1200 cm^{-1} for all the metals in the present study, representing C–H bending vibrations of CH_3, CH_2 and CH functional groups (Wolkers *et al.*, 2004). The peak shifts in the spectral region 1200–900 cm^{-1} corresponds to C–O, C–C, C–O–C and C–O–P stretching vibrations of polysaccharides. Changes of peaks in this region in the presence of all the four metals suggests interaction of cell surface polysaccharides with metal ions, facilitating their biosorption. A stretching peak of 2367 cm^{-1} is assigned to the asymmetric stretching of the isocyanate group (–N=C=O) (Gholap and Badiger, 2004). The yeast isolate demonstrated stretching of the peak at 2362 cm^{-1}, indicating the involvement of N=C=O group in the biosorption of Pb (Figure 4d). Broad spectral bands in the region 3700–3300 cm^{-1} demonstrate N–H or O–H stretching vibrations (Guo and Zhang, 2004), and alkyl chains are represented by other bands around 2920–2850 cm^{-1}. A peak shift in the region 3300 cm^{-1} was observed for all the metal-treated biomass, suggesting N–H or O–H stretching vibrations. Also, a peak shift around 2800 cm^{-1} represented –CH stretching vibrations of $–CH_3$ and $–CH_2$ groups during biosorption of these heavy metals.

Metal removal from the culture medium by the yeast isolate

A considerable percentage of heavy metals was removed from the culture supernatant by the yeast isolate. However, the metal uptake capacity varied with the initial concentration of the metal added to the solution, the highest being 98% removal in the presence of 10 mg/l Pb and Zn (Figure 5). Minimum removal was observed for the solution containing 200 mg/l Cu and was found to be 30% (Figure 5). The yeast *Lodderomyces elongisporus* was shown to remove 81% of Cu from the medium, added at a concentration of 0.1 mg/l after 96 h of incubation (Rehman *et al.*, 2008). Several other studies have also emphasized the role of bacteria (Shakoori and Muneer, 2002), fungi (Dönmez and Aksu, 2001) and algae (Feng and Aldrich, 2004) towards tolerance to, or bioaccumulation

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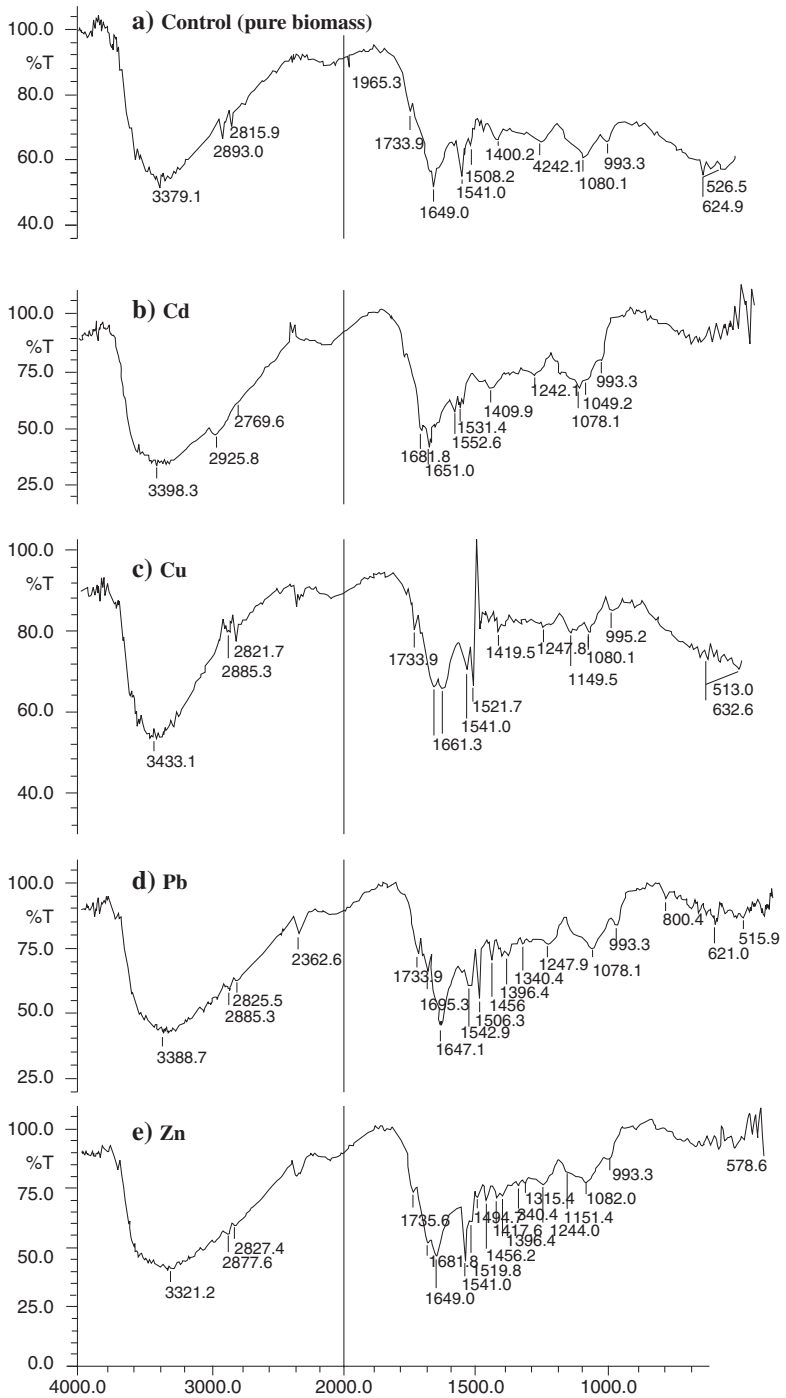

Figure 4. The FT-IR spectrum patterns of deep-sea *Cryptococcus* sp; control (a) and the biomass loaded with: (b) Pb; (c) Zn; (d) Cd and (e) Cu

of, Cu from metal solutions. Among fungi, most of the yeast isolates have been found to be sensitive to higher concentration of Cu, except for a deep-sea *Cryptococcus* sp. which could tolerate a 50 mM concentration of $CuSO_4$ (Abe *et al*., 2001). The results of the present study are also in

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accordance with the above observation where a deep-sea yeast tolerated ~10 mM concentration of $CuSO_4$ in solution and successfully removed ~80% of Cu from a solution containing 10 mg/l (~0.1 mM) (Figure 5). However, Abe *et al*. (2001) demonstrated the growth of yeast on a medium plate with 50 mM concentration of $CuSO_4$, whereas in the present study metal tolerance of yeast was shown in liquid media. The yeast may exhibit different growth patterns in the presence of metals on solid and liquid media.

The results of the present study suggest greater capability of this deep-sea isolate for different heavy metal removal from solutions, which may find application in the bioremediation of metal-contaminated sites. Further studies involving metabolic phases of this yeast after exposure to heavy metals are suggested for future research, which should better elucidate the understanding of biosorption mechanism. Up- and downregulated genes under simulated deep-sea conditions were studied in this isolate (Singh *et al*., 2012). It will be of interest to look for such genes in the presence of heavy metal stress and any commonality of such genes under combinations of these various stress conditions.

Conclusion

The deep-sea *Cryptococcus* sp. exhibited remarkable tolerance and biosorption towards different concentrations of the four heavy metals tested. The optimum temperature and pH range for its growth in the presence of metals was found to be 30°C and 6–8, respectively. Altered cell surface morphology in the presence of heavy metals was observed by SEM. The uptake of metals and the bonds involved in metal ion interaction onto the cell surface were demonstrated by EDAX and FTIR analysis, respectively. In addition, a high percentage of metals were removed from the culture medium by this isolate. All these results suggest that this deep-sea yeast displays significant metal tolerance and biosorption capabilities and may be used for the bioremediation of metal-contaminated sites. Being isolated from an environment rich in polymetallic nodules, this isolate may have evolved a defensive mechanism to detoxify the environment by bioaccumulation of heavy metals, and have developed tolerance to them. A detailed study on metabolic approaches adopted by this yeast during the metal uptake process may provide greater insight into its ecological role in such deep-sea sediments.

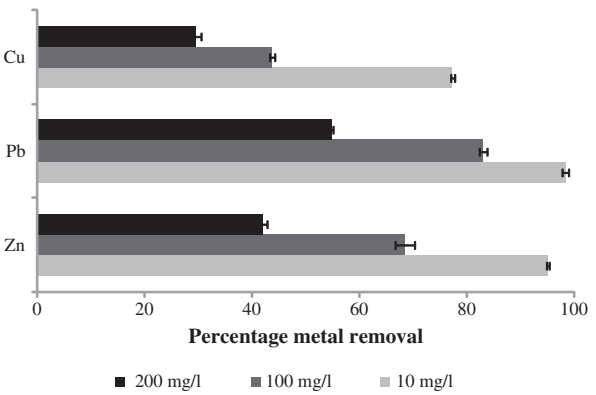

Figure 5. Percentage removal of metals from the culture filtrate by NIOCC#PY13 at three different concentrations of heavy metals. The results for percentage removal of Cd is not included, as the analysis could not be done due to non-availability of a Cd lamp in the spectrophotometer

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